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13. ABSTRACT *(Maximum 200 Words)*

The ISAC meeting was held during 24 - 28, June, 1999, in Philadelphia. Since its inception, these meetings were focussed on arterial chemoreceptors and their functions. This time, it was expanded to include oxygen sensing in other tissues and cells, and the genes involved in oxygen sensing. This genetic flavour made the meeting more exciting, and it was attended by more than two hundred participants at one time of the proceedings. The idea was to bring together scientists from cellular and systemic boundaries of physiology, working at the interface of cellular and molecular biology.

The evening of the 24th began with a reception and opening of the Symposium. The first two days were devoted to genes and genetic expression of oxygen sensing, and the next two days were devoted to oxygen sensing by arterial chemoreceptors and chemoreflexes. Altogether, there were sixteen sessions, and there were fourteen posters each day on the average. There was a round-table conference at the end of the second day. There were fourteen papers from the young investigators competing for the awards, four of which were judged winners by the audience: two for the Heymans-de Castro-Neil Awards, and two for Comroe, Forster & Lambertsen Awards.

One special feature of the Symposium was that twelve experts were asked to produce a volume of Respiration Physiology pertaining to the Symposium, and it was precirculated to the symposiasts. This volume gave a preview of the Symposium. The proceedings is in press: Plenum/Kluwer Press.

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(5) INTRODUCTION:

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There was a round-table conference at the end of the second day, discussing the genomic aspects. A similar round-table conference was originally planned to take place at the end of fourth day but by then there had been so much discussion that it was felt unnecessary.

The original gramaphone recordings of the carotid body sensory discharges which were made in the 1930's by Drs. Zotterman, von Euler and Liljestrand were played to the audience at the banquet. We are grateful to Professor curt von Euler, Karolinska Institut, Stockholm, Sweden, for the gift.

The council of the ISAC met, and at a business meeting the membership decided the venue of the next meeting which is to be in Lyon, France, in 2002, with Jean Marc Pequignot as its president. Tentatively the meeting after that is to be in held Kita-kyushu, Japan in 2005. There will be a section on Arterial Chemoreception at IUPS Meeting in New Zealand in 2001.

The Symposium was only possible because of the funds made available to us by generous gifts, particularly from the Bara Foundation, US Army Research Administration, Ecosystems Tech Transfer, Inc. and by Merck & Company. The Division of Lung Diseases: National Heart, Lung and Blood Institute, provided a conference grant (13-HL-60955). We are also fortunate to have received additional anonymous donations. We are grateful to them all.

Finally, we are grateful to the participants who came and contributed to the success of the Symposium. Special thanks are due to Mrs. Mary Pili (University of Pennsylvania, Philadelphia, Pa., USA) and Mrs. Marianne Sperk (Case Western Reserve University, Clevelend, Ohio, USA) and Mrs. Michelle Duparc, Ruhr-University at Bochum, Germany for their secretarial managements.

(7) KEY RESEARCH ACCOMPLISHMENTS:

Advanced knowledge as a result of the Symposium.

(8) REPORTABLE OUTCOMES:

Manuscripts in press (Plenum Press).

(9) CONCLUSIONS:

Genomics of oxygen sensing.

(10) REFERENCES:

(1) Respiration Physiology: Vol. 15: 115-260, 1999.
(2) Proceedings in Plenum Press.

(11) APPENDICES:

Enclosed are the list of articles that are in press.

(12) BINDING:

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(13) FINAL REPORTS:

All publications are listed.

(5) INTRODUCTION:

(6) BODY

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RESPIRATION PHYSIOLOGY

SPECIAL ISSUE:
Oxygen Sensing in the Body

GUEST EDITORS:
Sukhamay Lahiri
Helmut Acker

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INSTRUCTIONS TO AUTHORS

(Regularly updated on the Internet page of the journal: http://www.elsevier.nl locate resphysiol)

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Respiration Physiology publishes original articles or notes concerning the field of respiration in its broadest sense. Accordingly the journal covers all aspects, from the molecular to the organism level, of: control of breathing; mechanics of breathing; gas exchange in lungs, gills, skin, and tissues; acid-base balance; cell physiology of the respiratory tract; respiration at rest and exercise; respiration in normal and unusual conditions, like high or low pressure or changes of temperature, low ambient oxygen; embryonic and adult respiration; comparative respiratory physiology. Papers on clinical aspects, articles on original methods, as well as theoretical papers in respiratory physiology are also considered as long as they foster the understanding of respiratory physiology.

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Types of Publication. Aside from original research articles, *Respiration Physiology* features three other types of communication. A *Short communication* provides a rapid publication opportunity (usually 6–8 weeks from receipt of the accepted manuscript by the publisher) for short, concise papers dealing with original material within the scope of the journal. *Short communications* should not exceed 4 printed pages (about 8 manuscript pages), including up to 2 Figures, 1 Table and up to 10 References. Style of the manuscripts must otherwise conform with that of ordinary research articles (see below) and must, in particular, have an Abstract. The fax number of the corresponding author must be given on the title page. In addition, state-of-the-art reviews, prepared on invitation, will regularly appear under the title of *Frontiers review*. Furthermore, *Special Issues* are regularly published with the help of invited Guest Editors. These issues combine reviews on topics of particular scientific interest and can arise from scientific conferences. Suggestions for topics of *Special Issues* as well as for Guest Editors are invited by the Editor (see address below).

Authorship. Each author should have participated sufficiently in the work to justify authorship. This participation must include: (a) critically important intellectual contribution to the conception, design, and or analysis and interpretation; (b) drafting the manuscript or critically reading it; and (c) thorough reading and final approval of the version to be published. Participation solely in the collection of data or provision of funds, space or equipment does not justify authorship. All authors take public responsibility for the paper as a whole, i.e., conception and design, data, analysis, and interpretation.

The acknowledgement section should list (a) other contributors for whom authorship is not justified, e.g. technical help; (b) financial and material support.

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All manuscripts should be submitted to:

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Symbols and Units. The meaning of the symbols should be clearly understood from the context, and all symbols that are not commonly used should be defined on their first appearance in the Abstract **and** in the main text.

The symbols should conform with the glossary of terms and symbols in respiratory physiology proposed by the International Union of Physiological Sciences. The following are examples of main symbols and their modifiers. A more complete description of units, symbols and abbreviations is given in the Combined Index to Volumes 51–75.

Main symbols:

F	Fractional concentration in dry gas phase	$\dot{V}$	Volume per unit time, e.g. flow, ventilation
P	Gas pressure in general	C	Concentration
D	Diffusing capacity	S	Saturation
f	Frequency	R	Respiration exchange ratio
V	Volume		

Modifiers (small capitals and ordinary small letters, on the same line as main symbol):

I	inspired	L	pulmonary
E	expired	R	respiratory
ET	end-expired, end-tidal	b	blood in general
A	alveolar	a	arterial
T	tidal	c	capillary
D	dead space	c′	end-capillary
B	barometric	v	venous
H	heart, cardiac	$\bar{v}$	mixed venous blood

STPD	Standard temperature and pressure, dry (0°C, 760 mmHg)
BTPS	Body temperature and pressure, saturated with water vapor
ATPS	Ambient temperature and pressure, saturated with water

All symbols referring to gas species are in subscript, e.g. P_{CO_2}. Dash above a symbol designates a mean value, e.g. $P\bar{v}$.

Examples of combinations

$F_{E_{N_2}}$	Fractional concentration of nitrogen in dry expired gas
$P_{ET_{CO_2}}$	Partial pressure of CO_2 in end-tidal air
$P_{C_{O_2}}$	Partial pressure of oxygen in capillary blood (distinct from P_{CO_2})
$P_{A_{O_2}} - P_{a_{O_2}}$	Oxygen partial pressure difference between alveolar gas and arterial blood
P_B	Barometric pressure
$C_{v_{CO_2}}$	Concentration of carbon dioxide in venous blood
$S_{\bar{v}_{O_2}}$	Saturation of oxygen in mixed venous blood
$\dot{V}_E$	Expired ventilation (not minute ventilation or minute volume)
V_T	Tidal volume
f_R	respiratory frequency
f_H	cardiac frequency

continued on inside back cover

RESPIRATION PHYSIOLOGY

SPECIAL ISSUE:
Oxygen Sensing in the Body

GUEST EDITORS:
Sukhamay Lahiri (PA, USA)
Helmut Acker (Dortmund, Germany)

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Respiration Physiology 115 (1999) 115–116

Foreword

Oxygen sensing: molecule to man

The purpose of this special issue is to relate the functional adjustments to hypoxia at the genomic and systemic levels and to describe what is currently known of the cellular processes linking the two. It will bring together scientists from the cellular and systemic boundaries of physiology as well as investigators who work at the interface of cellular and molecular biology. Twelve scientists who will participate in the conference to be held in Philadelphia, PA, from 25–28 June 1999 have been asked to contribute to this special issue, which will be published before the symposium. It is thus hoped that this issue will provide us with the scientific flavor of the conference. The idea to produce this special issue evolved from discussion with Peter Scheid, Editor-in-Chief of this journal, to whom we are grateful. Our thanks also go to the contributors. The issue will be made available to attendees of the symposium, and in fact to all interested.

This multi-disciplinary nature of the conference will attract scientists and technologists, and the interaction among them is expected to produce future research ideas, particularly for the young participants. The need for such multi-disciplinary gathering, focusing on oxygen sensing, is obvious and timely.

Exposure to hypoxia produces a set of respiratory, circulatory, neural and metabolic responses that vary over time. It has become increasingly clear that these changing responses depend on a genomic infrastructure. Recent studies have demonstrated that hypoxia, in addition to elevating intracellular levels of calcium that leads to neurotransmitter release, can activate sets of genes that increase the formation of proteins. Among these are erythropoietin, tyrosin hydroxylase, the glycolytic enzymes, genes for nitric oxide synthase and heme oxygenase, and the gene encoding vascular endothelial growth factor. These gene sets form the genomic foundation of the physiologic adaptations to reduced oxygen levels.

The conference will include the topic of oxygen sensing by ion channels and by metabolic means, signal transduction in higher organisms, and mechanisms by which hypoxia regulates genes that impact on physiologic processes. This will deal with mammalian arterial chemoreceptors which, as a result of oxygen sensing, transduce neural impulses and, through central integration, evoke various reflex effects. Pulmonary myocytes produce local effects of hypoxia by constriction, leading to increases in pulmonary artery pressure, and improve the ventilation perfusion matching. The common element in all these is the restoration of cellular oxygen homeostasis.

With the advent of the development of cellular and molecular biological approaches, tremendous advances have been made in understanding oxygen sensing mechanisms in general and regulation of gene expression by oxygen in particular. Despite these advances, no meeting on genomics of oxygen sensing has been held in the recent decade. We believe that such a meeting is capable of facilitating the cross talk between the cellular, molecular and systemic physiologists working on oxygen sensing mechanisms.

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The conference itself is divided into two sessions: *Genomics of Oxygen Sensing* and *Arterial Chemoreception*. We will have about 60 oral presentations during the four days of the meeting. There will be two sessions on *Young Investigator Awards*. Because of the emphasis on interactions, there will be no parallel sessions. We will hold 1-h poster sessions each day, particularly for graduate students and post-doctoral trainees. Two Round Table Conferences will also be held.

The Symposium was only possible because of the funds made available to us by generous gifts, particularly from the Barra Foundation, US Army Research Administration, and by Ecosystems Tech Transfer, Inc. Also, Hoechst Marion Rousel and Merck & Company contributed. The National Institutes of Health, Division of Lung Diseases, National Heart, Lung and Blood Institute, provided us with a conference grant (13-HL-60955). We also are fortunate to have donations from individuals who wanted to remain anonymous.

Finally, special thanks are due to Mrs. Mary Pili, University of Pennsylvania, for expert secretarial assistance and to Mrs. Jane Kerr, Associate Publishing Editor, Elsevier Science B.V.

Sukhamay Lahiri
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March 1999

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Respiration Physiology 115 (1999) 117

Editorial

Oxygen sensing in the body

A number of special issues have been published by *Respiration Physiology* in the past. They have largely emerged from scientific meetings where they compiled the major presentations. Special issues have, thus, served as comprehensive overviews of certain 'hot' areas in respiratory physiology.

Respiration Physiology has now expanded this format, and several special thematic issues are planned to be published as part of the annual publication schedule of the journal. Extra effort will be afforded to the promotion of these special issues to ensure that they receive the maximum breadth of exposure. Thus, *Respiration Physiology*, together with its *Frontiers review* series, will amplify its information service on '*What is Hot in Respiratory Physiology?*'

The present special issue is unique in that it does not contain presentations from a meeting which has already taken place, rather it combines papers which will be presented at a forthcoming meeting. It thereby provides information for those participants who want to prepare themselves for a more effective meeting, while serving those who cannot attend but wish to be updated on a particular field.

The special issue combines papers from leading scientists in the field of cellular oxygen sensing and presents our knowledge as well as controversies regarding single components of the oxygen sensing signal cascade. This involves heme proteins, reactive oxygen species, ion channels, transcription factors, genes and protein synthesis.

The special issue is thus designed to set the stage for further discussion in the symposium on oxygen sensing which will take place within the *XIV International Symposium on Arterial Chemoreception*, to be held in Philadelphia, PA, from 24–28 June 1999.

My thanks are due to the Guest Editors, Dr Sukhamay Lahiri and Dr Helmut Acker, for their invaluable help with this issue. They have contributed the list of authors, and it is thus to their credit that the topic of oxygen sensing is dealt with in a breadth that goes far beyond the field of respiration *sensu strictiori*. Their input into the editing of the issue is also gratefully acknowledged. Thanks are due also to Jane Kerr, Associate Publishing Editor, Elsevier Science B.V., without whose help this issue could not have been produced in the limited time available. Finally, I wish to acknowledge the invaluable and indefatigable assistance of my secretarial staff, in particular Michelle Duparc.

Peter Scheid
Bochum
Germany

February 1999

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Respiration Physiology 115 (1999) 119–133

Models for oxygen sensing in yeast: implications for oxygen-regulated gene expression in higher eucaryotes

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Abstract

Adaptation to changes in oxygen tension in cells, tissues, and organisms depends on changes in the level of expression of a large and diverse set of proteins. It is likely that most cells and tissues possess an oxygen sensing apparatus and signal transduction pathways for regulating expression of oxygen-responsive genes. Although progress has been made in understanding the transcriptional machinery involved in oxygen-regulated gene expression of eucaryotic genes the underlying mechanism(s) of oxygen sensing and the signaling pathways that connect oxygen sensor(s) to the transcription machinery of eucaryotes are still poorly understood. The yeast *Saccharomyces cerevisiae* is ideal for addressing these problems. Indeed, it is well-suited for broadly based studies on oxygen sensing at the cellular level because it lends itself well to genetic and biochemical studies and because its genome has been completely sequenced. This review focuses on oxygen-regulated gene expression and current models for oxygen sensing in this yeast and then considers their applicability for understanding oxygen sensing in mammals.

Keywords: Oxygen tension; Eucaryotic; Mammals

1. Introduction

With the exception of obligate anaerobes all organisms depend on oxygen for the production of metabolic energy and for some biosynthetic reactions. Molecular oxygen is uniquely suited for its role in metabolic energy production because it has a high redox potential, which permits maximal energy conservation from reduced substrates. Although molecular oxygen is chemically inert (i.e. kinetically stabilized) it can, in the presence of appropriate paramagnetic activators, give rise to unstable reactive oxygen species (ROS) (superoxide, hydrogen peroxide, hydroxl ion) that are harmful to cells (Cadenas, 1989). Thus, although aerobic cells use oxygen they must also protect themselves from the harmful reactive oxygen spe-

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cies that are sometimes produced from it. The growing evidence linking reactive oxygen species to degenerative disease and aging (Ames et al., 1993; Shigenaga et al., 1994; Irani et al., 1997) has increased the importance of understanding how cells 'sense' and respond to changes in oxygen concentration in their environment. Cells, tissues, and organisms must also sense when oxygen is limiting and respond accordingly. The adaptation of cells and organisms to different oxygen concentrations requires a complex biochemical program that affects the intracellular levels and activities of a number of proteins (Bunn and Poyton, 1996; Hochachka et al., 1996). When most organisms are shifted from one oxygen tension to another they exhibit both a short-term and a long-term response. The short-term response (termed an acute response, Hochachka, 1998) is essentially immediate and requires no new gene expression. It is during this phase that cells sense the change in oxygen concentration and initiate a series of signal transduction pathways that will allow the organism to cope with its new oxygen environment. The long-term response (termed the acclimatory response, Hochachka, 1998) occurs over a longer time period and, in most cases, requires the expression of new genes whose products allow the organism to acclimate to the new oxygen tension. It is this 'long term' oxygen-dependent gene expression that will be considered here.

2. Examples of oxygen-dependent gene expression in procaryotes and eucaryotes

2.1. Mammals

A good example of an adaptive response to a change in oxygen tension comes from considering adaptation to hypoxia in mammals. The ability of mammals to respond to hypoxia is crucial to their survival. Moreover, their failure to respond properly can lead to diseases like anemia, myocardial infarction, retinopathy, and to the growth of tumors (Guillemin and Krasnow, 1997).

The response of mammalian cells to hypoxia ranges from immediate changes in energy metabolism to the activation of gene expression pathways. When mammals are first exposed to hypoxic or anoxic conditions there is an immediate switch from aerobic to anaerobic metabolism and a suppression of energy utilizing reactions (Hochachka et al., 1996). This is part of the acute response mentioned above. Upon longer exposure to hypoxia mammals and other vertebrates attempt to increase whole body ventilation and increase total red blood cell mass (Bunn and Poyton, 1996). At least five different oxygen responsive systems are involved (reviewed by Hochachka, 1998). First, carotid body oxygen sensors initiate an hypoxic ventilatory response that functions to compensate for acute oxygen shortage. This results in increased oxygen uptake by the lung. Second, oxygen sensors in the pulmonary vasculature initiate a pulmonary vasoconstrictor response designed to adjust lung and ventilation perfusion and blunt pulmonary vasoconstriction. This results in an increased efficiency of gas exchange in the lung. Third, oxygen sensors in the vasculature of other tissues induce the expression of VEGF1 (vascular endothelial growth factor 1) and its receptor, which function to promote angiogenesis and hence, expanded blood volume. This results in improved oxygen carrying capacity in the heart and probably the brain. Fourth, oxygen sensors in the kidney and liver induce the expression of erythropoietin, which functions to stimulate erythropoiesis. And fifth, hypoxia affects the expression of a wide variety of genes that affect oncogenesis, glucose transport, glycolysis, respiration, gluconeogenesis (Arany et al., 1996; Bunn and Poyton, 1996; Hochachka et al., 1996). Hypoxia also affects cell growth and division by inducing p53, which controls the G1 phase of the cell cycle (An et al., 1998). As discussed below, each of these response systems involves some type of 'oxygen sensor' and, in most cases, a signal transduction pathway that induces the expression of genes whose products function under hypoxic conditions. Some of these responses are common to all hypoxic cells, suggesting that every cell has its own oxygen-sensing apparatus. Indeed, the expression of hypoxic genes in many different tissues is activated by the transcription factor, HIF-1 (Bunn et al., 1998; Ratcliffe et al., 1998). Other responses (e.g. those

which take place in the carotid body) are mediated by a more centralized oxygen-sensing apparatus that keeps track of oxygen levels in the body and causes systemic changes in the delivery of oxygen to cells and tissues.

2.2. Bacteria

In the bacteria *E. coli* and *Salmonella*, oxygen availability determines whether energy will be produced by aerobic respiration, anaerobic respiration, or fermentation. This is possible because these organisms possess several different types of respiratory chains, which use different electron acceptors and terminal oxidoreductases (Lin and Iuchi, 1991; Poole, 1994; Bunn and Poyton, 1996). In the presence of oxygen *E. coli* uses one of two terminal oxidases, cytochrome bo oxidase or cytochrome bd oxidase, depending on the oxygen concentration. Cytochrome bo is used when cells are grown in atmospheric oxygen but is replaced by cytochrome bd oxidase when oxygen becomes limiting. In the absence of oxygen and the presence of an appropriate alternative electron acceptor *E. coli* cells can produce one of at least five alternative oxidoreductases. When supplied with the appropriate exogenous electron acceptors *E. coli* cells curtail the fermentative process in favor of respiration, and when given a choice of electron acceptors they preferentially use the acceptor with the highest midpoint potential (E'^0), thereby maximizing the utilization of energy that is available in reduced substrates. For example, *E. coli* favors O_2 ($E'^0 = +820$ mV) over nitrate ($E'^0 = +430$ mV), and nitrate over fumarate ($E'^0 = +30$ mV). This remarkable phenomenon suggests that these cells choose the most energetically favorable pathway for energy production. This ability to maximize energy production is brought about largely at the level of transcription and is affected by the oxygen-regulated repression or de-repression of genes that encode the different oxidoreductases. It is now clear that the relative levels of these oxidoreductases are affected through two transcription factors, Fnr and ArcA (Bunn and Poyton, 1996). Fnr is a global regulator of a large number of *E. coli* genes (Lin and Iuchi, 1991; Spiro and Guest, 1991). It functions in cells grown under anaerobic conditions and may act as either a transcriptional activator or repressor. Currently, it is not clear how Fnr senses and responds to anaerobiosis. ArcA is the target (i.e. regulatory component) in a two component signalling system (Iuchi et al. 1990; Iuchi and Lin, 1992). The sensor (i.e. upstream component) is ArcB, a kinase which autophophorylates itself in cells grown aerobically (Kato et al., 1997). Once phosphorylated, ArcB transphosphorylates ArcA, which may then function as either an activator or repressor of transcription. The stimulus for ArcB phosphorylation does not appear to be oxygen itself but has been attributed to either respiration or the redox state of the cytosol (Iuchi et al., 1990; Lin and Iuchi, 1994).

Another example of an oxygen-regulated gene expression pathway in procaryotes comes from the FixL-FixJ two component regulatory system in *Rhizobium meliloti*. The FixL protein is the 'oxygen sensor' (Monson et al., 1992). It is a hemokinase that phosphorylates the transcription factor FixJ under microaerophilic conditions. Once phosphorylated, FixJ functions as a transcriptional activator that upregulates two genes, nifA and fixK. Several studies have revealed that the kinase activity of FixL is affected by the heme group and that it is the spin state of the heme iron that is critical for the crosstalk between the heme and kinase domains (Gilles-Gonzalez et al., 1994, 1995; Bertolucci et al., 1996).

2.3. Yeast

As in bacteria and mammals, yeast cells are profoundly affected by oxygen. Some yeasts, like *S. cerevisiae*, are facultative anaerobes. In the presence of oxygen this yeast respires but under anaerobic conditions it supports its energy needs by fermentation. The intracellular levels and often activities of a large number of proteins in *S. cerevisiae* are affected by oxygen (Poyton and Burke, 1992; Zitomer and Lowry, 1992; Kwast et al., 1998). They include respiratory chain proteins as well as enzymes involved in the synthesis of heme, sterols, and unsaturated fatty acids. In addition, oxygen affects the levels of enzymes involved in the oxidative stress response and the

level of a translational initiation factor. The effects of oxygen on the intracellular concentrations of many of these proteins has been shown to be exerted through transcription of their genes. Most of these oxygen-regulated genes can be placed into one of two groups: aerobic genes, which are transcribed optimally in the presence of air; and hypoxic genes, which are transcribed optimally under anoxic or microaerophilic conditions. Oxygen may also affect expression via translational control, especially for proteins encoded by mitochondria DNA. In addition, oxygen may have indirect affects on the expression of some proteins. For example, because oxygen is required for heme biosynthesis in yeast and other eucaryotes (Labbe-Bois and Labbe, 1990), oxygen tension affects the folding or assembly of hemoproteins by affecting the availability or redox state of their prosthetic groups.

A striking example of the influence of oxygen on the level and activity of an exzyme comes from considering the effects of oxygen on the expression of yeast cytochrome *c* oxidase genes and the function of its oxygen-regulated isoforms (Poyton and McEwen, 1996; Burke and Poyton, 1998). This enzyme contains polypeptide subunits encoded by both nuclear and mitochondrial genomes. The three largest subunits (I, II and III) are encoded by mitochondrial genes (*COX1*, *COX2*, and *COX3*); they form the catalytic core of the enzyme. Oxygen affects the expression of two of these genes (*COXI* and *COX2*) post-transcriptionally. The other polypeptide subunits are encoded by nuclear genes; some of them modulate catalysis, whereas others function in the assembly or stability of the holoenzyme. Active preparations of yeast cytochrome *c* oxidase contain at least six subunits (IV, Va or Vb, VI, VII, VIIa and VIII) encoded by nuclear *COX* genes (*COX4*, *COX5a or COX5b*, *COX6*, *COX7*, *COX9*, and *COX8*, respectively). *COX5a* and *COX5b* encode interchangeable isoforms, Va and Vb, of subunit V (Trueblood and Poyton, 1987). The other nuclear-encoded subunits are encoded by single copy genes. Oxygen affects the expression of all of these nuclear genes at the level of transcription. All of these genes, except COX5b, are aerobic genes expressed optimally in the presence of air; they are turned off at very low oxygen concentrations (0.25–0.5 μM) (Burke et al., 1997, 1998). Conversely, *COX5b* is a hypoxic gene which is repressed by air and turned on at very low oxygen concentrations (0.2 μM) (Burke and Poyton, 1998; Burke et al., 1998). This inverse regulation of *COX5a* and *COX5b* by oxygen is especially interesting because these isoforms have differential effects on holocytochrome *c* oxidase activity. By altering an internal step in electron transport between heme a and the binuclear reaction center (composed of heme a_3 and Cu_B), the 'hypoxic' isoform Vb, enhances the catalytic constant (i.e. Turnover Number) 4-fold relative to the 'aerobic' isoform, Va (Waterland et al., 1991; Allen et al., 1995). Hence, these subunit isoforms allow cells to assemble functionally different types of holocytochrome c oxidase in response to different oxygen concentrations.

2.4. Oxygen-regulated gene expression

From each of the above examples, it is clear that oxygen is an important environmental signal and that changes in its availability can initiate a complex series of events that begins with some sort of sensor and ends with a signalling pathway that either up or down-regulates a number of genes. Thus far, the best understood oxygen sensor(s) and signalling pathways are those in procaryotes. Although progress has been made in understanding the transcriptional machinery involved in oxygen-regulated gene expression of eucaryotic genes the underlying mechanism(s) of oxygen sensing and the signalling pathways that connect oxygen sensor(s) to the transcription machinery in eucaryotes are still poorly understood. The yeast *Saccharomyces cerevisiae* is ideal for addressing these problems. Indeed, it is well-suited for broadly based studies on oxygen sensing at the cellular level because it lends itself well to genetic and biochemical studies and because its genome has been completely sequenced. In the following sections oxygen-regulated gene expression and current models for oxygen sensing in this yeast will be reviewed, and their applicability for understanding oxygen sensing in mammals will be considered.

3. Oxygen-regulated transcription in yeast

So far, only a few transcription factors have been implicated in the oxygen-regulated transcription of *S. cerevisiae* genes (Fig. 1). Three of these function to regulate the expression of aerobic genes. Hap1 and Hap2/3/4/5p activate expression of aerobic genes when cells are grown in the presence of air and have no effects when cells are grown in the absence of air. Hap1p functions as a homodimer to activate oxygen-dependent expression of *CYC1*, *CYB2*, *CYT1*, *COR2*, *HEM13*, *HMG1*, *CTT1*, *YHB1*, *ROX1* and *TIF51A* (Kwast et al., 1998). It is not yet known if Hap1p is a conserved transcription factor present in other eucaryotes. Hap2/3/4/5p is a tetramer that binds to CCAAT promoter elements. It activates the oxygen-dependent expression of at least three aerobic genes (*COX5a*, *COX4*, and *QRC8*) in a carbon source-dependent manner. Three of its polypeptide subunits (Hap2p, Hap3p, and Hap5p) are functional homologues of three subunits (NF-YA, NF-YB, and NF-YC, respectively) of mammalian NF-Y (Sinha et al., 1995). The assembly and DNA binding activity of NF-Y is regulated by cellular redox (Nakshatri et al., 1996). Neither Hap1p nor Hap3/3/4/5p activate the expression of their target genes in the absence of air, or in heme-deficient cells (Fig. 1). At least one additional transcription factor, HDS-binding factor, regulates expression of aerobic yeast genes in a heme (and oxygen)-dependent fashion (Trawick et al., 1992; Bunn and Poyton, 1996). This regulates expression of at least two aerobic genes (*COX6*

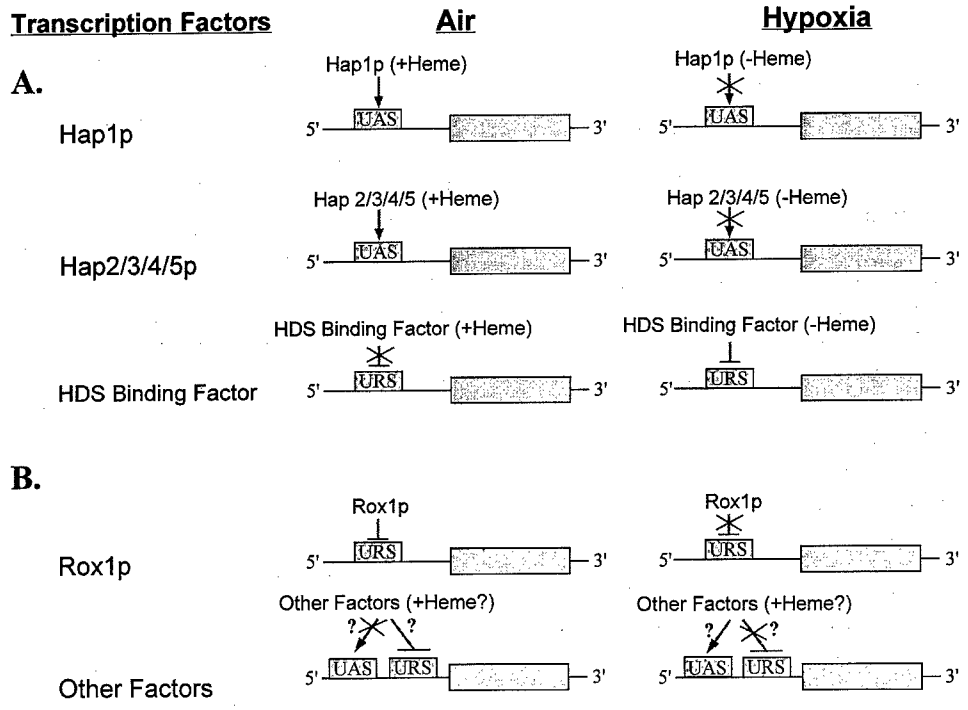

Fig. 1. Transcription factors involved in oxygen-regulated gene expression in yeast. Panel A: transcription factors that function to regulate aerobic genes. Hap1p and Hap2/3/4/5p work positively through UAS *cis* elements. Both require cells with a functional heme biosynthetic pathway. The HDS factors work negatively through URS *cis* elements. In the presence of air Hap1p or Hap2/3/4/5p activate transcription but at low oxygen concentrations their ability to up-regulate their target genes is compromised. In contrast, the HDS-binding factor(s) (which have not yet been identified) function to de-repress expression of their target genes in aerobic cells and repress them at low oxygen concentrations. Panel B: transcription factors that function to regulate hypoxic genes. Rox1p represses expression of its target genes in aerobic cells but derepresses their expression at low oxygen concentrations. Other factors, which have not been identified yet, may function to either activate or derepress expression of hypoxic genes in low oxygen.

and *SOD2*). The *cis*-acting promoter elements that mediate this act negatively; when these elements are deleted transcription is activated. In contrast to Hap1p and Hap2/3/4/5p this factor de-represses expression of its target genes when cells are grown in air and represses their expression in hypoxic and/ or anaerobic cells (Fig. 1).

The best understood transcription factor involved in the oxygen-regulated expression of hypoxic yeast genes is Rox1p (Reo1p), which represses the transcription of these genes under aerobic conditions (Zitomer and Lowry, 1992; Pinkham and Keng, 1994; Bunn and Poyton, 1996; Kwast et al., 1997). Genes that are regulated by Rox1p include: *COX5b*; *CYC7*; *AAC3*; *HEM13*; *HMG1*; *HMG2*; *ERG11(14DM)*; *CPR1 (NCP1)*, *OLE1*, and *ANB1* (Kwast et al., 1998). Because *ROX1* is an aerobic gene whose expression is activated by Hap1p it is not expressed under hypoxic conditions. Hence, under conditions of low oxygen its target genes are de-repressed (Fig. 1). There is accumulating evidence for the involvement of other factors, besides Rox1p, in the regulation of hypoxic yeast genes (Kwast et al., 1998). These repress transcription of hypoxic genes in a Rox1p-independent manner. Some of these appear to be required for expression of Rox1p-dependent genes (*CYC7* and *ANB1*) while others affect expression of Rox1p-independent genes (*DAN1*, *GPD2*, and *SRP1*). It is not yet clear if these bind to UAS elements, and function as activators, or to URS elements and function as repressors (Fig. 1).

4. Oxygen sensing and signal transduction in yeast

4.1. Involvement of heme

It is well established that the heme biosynthetic pathway is required for the expression of both aerobic and hypoxic yeast genes and it has been argued that heme itself (i.e. Fe-Protoporphyrin IX) is an effector molecule used in monitoring oxygen tension (Zitomer and Lowry, 1992; Bunn and Poyton, 1996). This is based on the following observations. (1) Oxygen is required for two steps in heme biosynthesis, the formation of protoporphyrinogen IX by coproporphrinogen oxidase III and the formation of protoporphyrin IX by protoporphyrinogen oxidase IX oxidase (Labbe-Bois and Labbe, 1990). (2) The step catalyzed by coproporphyrinogen oxidase III is rate limiting; hence, it could function to match heme concentration to oxygen tension. (3) Heme deficiency, in mutants with defects in the heme biosynthetic pathway, mimics the effects of oxygen deprivation on transcription; that is, aerobic genes are downregulated and hypoxic genes are upregulated in heme-deficient mutants (Zitomer and Lowry, 1992). The most often used mutant, *hem1*, lacks δ-ALA synthase. This mutation can be bypassed by the addition of δ-aminolevulinate, the product of δ-ALA synthase. So, *hem1* cells grown in the presence of δ-aminolevulinate have normal levels of heme whereas those grown in its absence have reduced levels of heme. (4) Supplementation of anaerobic cells with heme reverses the effects of anaerobiosis on both aerobic and hypoxic genes (Lowry and Lieber, 1986; Hodge et al., 1989).

Although it is not yet clear how heme functions in oxygen sensing and signal transduction two fundamentally different models have been proposed (Poyton and Burke, 1992; Bunn and Poyton, 1996).

4.1.1. Model 1

In the first model, heme would serve as a redox-insensitive ligand that binds to a transcription factor (Zitomer and Lowry, 1992; Pinkham and Keng, 1994), as has been proposed for the heme-dependent activation of Hap1p (Zhang and Guarente, 1994, 1995). As a hydrophobic molecule, heme would be analogous to a steroid hormone and the transcription factor, Hap1p, would be analogous to a steroid hormone receptor. This model for the involvement of heme in oxygen-regulated gene expression assumes that heme concentration is affected by oxygen concentration, that heme per se functions to regulate the activity of the trancription factor Hap1p, and that heme is a redox-insensitive cofactor for Hap1p. Hap1p and heme activate aerobic genes, including *ROX1*. In the presence of air *ROX1* would be expressed and its product, ROX1p, would func-

tion as a repressor of many hypoxic genes. According to this model, transcription of aerobic genes would be activated under aerobic or heme-proficient conditions while transcription of hypoxic genes would be repressed. Under anoxic or heme-deficient conditions, transcription of aerobic genes would not be activated and transcription of hypoxic genes would be de-repressed (due to loss of repression by Rox1p or other transcription factors). In this mode of operation, heme concentration and not its redox state per se is important. This type of sensing pathway would be useful for low oxygen concentrations that approach the affinity constant (K_m) (< 0.1 μM O_2) for oxygen binding to coproporphyrinogen oxidase III, a limiting step in heme biosynthesis (Labbe-Bois and Labbe, 1990), because it would provide an efficient 'on-off' switch for transcription.

4.1.2. Model 2

In the second model, heme itself would function as a redox-sensitive prosthetic group in a transcription factor (or an upstream 'oxygen sensor'). In this mode of operation it is the redox or spin state of heme but not its concentration that would be important. A pathway like this would be useful for a range of oxygen concentrations that is above the K_a for oxygen binding to coproporphyrinogen oxidase III because heme concentration per se should not be affected in the range. At present, it is not known if any of the transcription factors involved in oxygen-regulated expression of yeast genes are redox-sensitive hemoproteins. Indeed, although there is evidence that Hap1p binds heme, it is not known if its heme moiety mediates redox-dependent control of its transcriptional activity (Zhang and Guarente, 1995). However, as discussed below there is evidence for the involvement of the hemoprotein cytochrome *c* oxidase in the induction of a subset of hypoxic yeast genes (Kwast et al., 1999).

4.2. Oxygen concentration dependent expression of aerobic and hypoxic genes

One obvious question concerning how cells sense oxygen is whether oxygen-regulated genes respond in an 'all-or-none' fashion to the presence of oxygen or whether they respond in a graded fashion to different oxygen concentrations. Indeed, dose–response curves that relate gene expression to oxygen concentration are of fundamental importance because they can place limits on possible models for oxygen sensing. They can also provide an experimental paradigm for examining how the molecular components of oxygen sensing pathways function. Recently, the author's laboratory has studied the effects of oxygen concentration on the expression of a number of aerobic and hypoxic yeast genes (Burke et al., 1997, 1998; Kwast et al., unpublished). These studies have revealed several new insights concerning oxygen-regulated gene expression in yeast. First, they have demonstrated that the level of expression of aerobic genes is determined by the concentration of oxygen and not merely its presence or absence. Second, they have revealed that hypoxic genes are regulated more tightly than aerobic genes with respect to the oxygen concentration over which they are expressed. Third, they show that aerobic genes are induced faster than hypoxic genes following a shift in oxygen concentration. Fourth, they reveal that transcripts from both aerobic and hypoxic genes are turned over when cells are shifted from one oxygen concentration to another. And fifth, they reveal two distinct classes of aerobic genes; those aerobic genes that have hypoxic gene counterparts have different dose–response curves to oxygen concentration than aerobic genes that do not have hypoxic gene counterparts (Burke et al., 1997; Kwast et al., 1998).

The dose–response curves relating the steady state mRNA levels from aerobic yeast genes to oxygen concentration are complex, exhibiting two phases (Burke et al., 1997; Kwast et al., unpublished). The first phase (Phase I) is between oxygen concentrations of 200 and 0.5 μM O_2, while the second phase (Phase II) is below 0.5 μM O_2 (Fig. 2). For an aerobic gene that has a hypoxic gene counterpart, the level of expression stays constant during Phase I but drops off sharply during Phase II. For an aerobic gene that lacks a hypoxic gene counterpart, there is a gradual decline in transcript level during Phase I, and a sharp decline in transcript level as the oxygen

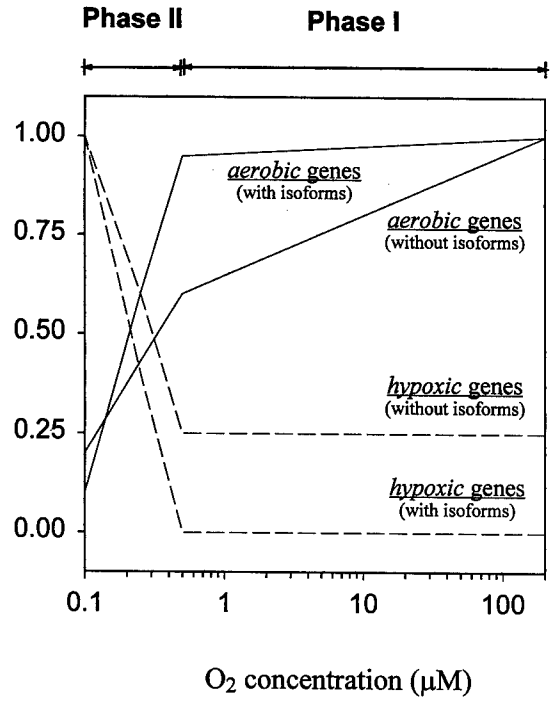

Fig. 2. Typical dose–response curves relating oxygen concentration to transcipt levels from aerobic genes with isoforms, aerobic genes without isoforms, hypoxic genes with isoforms, and hypoxic genes without isoforms. See text for details. Taken from Burke et al. (1997, 1998), Kwast et al. (1998 and unpublished).

concentration drops in Phase II. The dose–response curves for hypoxic genes are also biphasic (Fig. 2). Hypoxic genes that have an aerobic counterpart are essentially 'off' until very low oxygen concentrations (0.5 μM O_2) are reached. In contrast, hypoxic genes that do not have aerobic counterparts are expressed at detectable levels at all oxygen concentrations but are turned up at low oxygen concentrations, below 0.5 μM O_2. From the biphasic nature of these dose–response curves it appears that the oxygen concentration at the break between Phase I and Phase II represents an oxygen threshold.

These findings have important implications for the two models for oxygen sensing described above. According to Model 1 heme concentration alone is responsible for the level of expression of oxygen-regulated yeast genes. For oxygen concentrations near the affinity constants for oxygen binding to coproporphyrinogen III or protoporphyrinogen IX oxidase (where the oxygen concentration would be expected to affect heme levels) heme concentration could provide an efficient 'on-off' switch for transcription. Because these affinity constants are low (< 0.1 μM O_2 for yeast coproporphyrinogen II oxidase; Labbe-Bois and Labbe, 1990), this type of model could explain the low oxygen thresholds observed here for the expression of both aerobic and hypoxic genes. Model 1 could also explain the oxygen-concentration-dependent expression of aerobic genes at oxygen concentrations that are below these thresholds (i.e. Phase 2 of the dose–response curves). At these oxygen concentrations a small change in oxygen concentration leads to a large change in expression. Model 1 can not explain the oxygen concentration-dependent increase in expression observed for aerobic genes between 1 and 200 μM O_2 (Phase 1) because heme concentration per se should not be affected at these oxygen concentrations (which are well above the K_m for oxygen binding to coproporphyrinogen oxidase III or protoporphyrinogen IX oxidase). This part of the dose–response curve for aerobic genes is more easily explained by Model 2, in which a redox-sensitive hemoprotein senses oxygen levels.

4.3. Involvement of the respiratory chain and cytochrome c oxidase in the induction of some hypoxic genes

Recently, the author's laboratory has asked if redox-sensitive hemoprotein(s), function in oxygen sensing in yeast (Kwast et al., 1999) by studying the effects of ligands that affect the oxygen binding properties of hemoproteins. First, the authors examined the effects of carbon monoxide (CO) on the expression of hypoxic genes were examined after shifting cells from normoxic to anoxic conditions. Carbon monoxide has remarkable specificity in biological systems, binding noncovalently to ferrous heme groups in a small number of hemoproteins. In mammalian cells CO markedly reduces or blocks the induction of hypoxic genes, a finding that is consistent with the involvement of the redox or spin state of a hemoprotein in the oxygen sensing pathway for these

genes (Goldberg et al., 1988; Goldberg and Schneider, 1994). In yeast, CO affects the induction of a subset of hypoxic genes. It completely inhibits the induction of *OLE1* and *CYC7* and partially inhibits the induction of *COX5b*. In contrast, CO has no effect on the induction of at least seven other hypoxic genes (*HEM13*, *HMG1*, *HMG2*, *ERG11*, *CPR1*(*NCP1*), *ANB1* and *AAC3*). *OLE1* and *CYC7* are also induced in aerobic cells by cobalt, a transition metal which when incorporated into heme affects its ability to bind oxygen (Bunn and Poyton, 1996). Together, these findings suggest that the redox state of a hemoprotein is involved in controlling the expression of at least two hypoxic genes. By using mutants deficient in each of the two major yeast CO-binding hemoproteins (cytochrome *c* oxidase and flavohemoglobin), it has been found that cytochrome c oxidase but not flavohemoglobin is required for the anoxic induction of *OLE1* and *CYC7*. And by using respiratory inhibitors as well as *cob1* and rho^0 mutants it has been found that the respiratory chain is involved in the hypoxic induction of these genes (Kwast et al., 1999). These findings suggest that electron transport through the respiratory chain is important for the hypoxic induction for *OLE1* and *CYC7* and that cytochrome *c* oxidase is probably the oxygen sensor that is involved. They also indicate that there are at least two classes of hypoxic genes in yeast (CO-sensitive and CO-insensitive) and that multiple mechanisms/pathways are involved in modulating the expression of hypoxic yeast genes.

The requirement of a functional respiratory chain and cytochrome *c* oxidase for the anoxic induction of *OLE1* and *CYC7* is puzzling because yeast cells grown under anaerobic conditions lack both cytochrome *c* oxidase and a functional mitochondrial respiratory chain (Linnane and Haslam, 1970). Moreover, cyanide inhibits the induction of both *OLE1* and *CYC7*, under anaerobic conditions (Kwast et al., 1999). These findings indicate that the 'signal' produced by the respiratory chain can be generated in the absence of oxygen.

4.4. Conclusions

Until recently, most studies on oxygen sensing and oxygen-regulated gene expression in yeast have focussed primarily on the *trans*-acting factors and *cis* sites that regulate the transcription of oxygen-responsive genes. These studies have identified heme as a central component of the oxygen sensing signal transduction pathway and have produced a popular model, Model 1, which explains how heme functions in oxygen sensing in yeast. This model assumes that heme concentration is affected by oxygen concentration, that heme per se functions to regulate the activity of transcription factors, and that heme is a redox-insensitive cofactor for these transcription factors (Zitomer and Lowry, 1992; Pinkham and Keng, 1994). According to this model Hap1p, Hap2/3/4/5p, or HDS-binding factor (together with heme) up-regulate aerobic genes. The level of activation would be related to heme concentration. Moreover, Hap1p activates *ROX1*, which encodes the Rox1p repressor. In the presence of air *ROX1* is expressed and its protein product, Rox1p, functions as a repressor of many hypoxic genes.

Recent findings have cast doubt on this simple model for oxygen sensing. First, the level of expression of aerobic genes is determined by the concentration of oxygen and not merely its presence or absence (Burke et al., 1997). Indeed, the level of expression of several aerobic genes that are under Hap1p control varies with oxygen concentration over a range of oxygen concentrations (200–0.5 μM O_2) where intracellular heme levels are not expected to vary (Burke et al., 1997). In addition, the oxygen dose–response curves for aerobic genes that have aerobic counterparts are different than those for aerobic genes that lack hypoxic counterparts (Burke et al., 1997; Kwast et al., unpublished). Insofar as Hap1p regulates aerobic genes of both types this finding suggests that other things besides Hap1p and heme concentration are involved. Second, the kinetics of induction of different hypoxic genes are widely divergent during a shift from aerobic to anaerobic growth (Kwast et al., 1999 and unpublished). This would not be expected if Rox1p functions as a universal repressor and its activity is determined only by its intracellular level. Moreover, it is now clear that other factors besides Rox1p are in-

volved in oxygen-regulated expression of hypoxic genes (see above). Finally, a redox-responsive CO-binding hemoprotein is involved in the anoxic induction (i.e. de-repression) of some, but not all, yeast hypoxic genes that are repressed by Rox1p (Kwast et al., 1999). This hemoprotein is likely to be cytochrome *c* oxidase (Kwast et al., 1999). This finding clearly indicates that there are at least two classes of hypoxic genes in yeast.

These new findings suggest that multiple pathways/mechanisms are involved in oxygen sensing in yeast and provide evidence for multiple classes of both aerobic and hypoxic genes. They also support the conclusion that heme may function by two fundamentally different mechanisms during oxygen sensing. In one mechanism (Model 1) oxygen concentration affects heme concentration and heme is a 'signal'. In the other mechanism (Model 2) heme is part of a hemoprotein and the redox and/or spin state of the heme signals the oxygen concentration seen by cells. At low oxygen concentrations intracellular heme concentration (Model 1) could dictate the level of expression of oxygen-regulated genes while at higher oxygen concentrations heme redox or spin state (Model 2) could dictate their level of expression.

5. Comparison of oxygen sensing and signal transduction in yeast and mammals

In trying to determine if either of the above models for oxygen sensing and signal transduction in yeast are generally applicable to eucaryotes it is useful to compare them to models derived from another well-studied system, mammalian cells. In the following sections we first compare the oxygen-responsive transcription machinery in yeast and mammalian cells. Then, we compare models for oxygen sensing/signal transduction in both yeast and mammals.

5.1. Transcriptional regulation

Although mammals, like yeast, have aerobic and hypoxic genes (Hochachka et al., 1996) little is known about the oxygen-regulated transcription of their aerobic genes. Indeed, studies on oxygen-regulated expression of mammalian genes have focused on the oxygen-regulated expression of hypoxic genes. These studies have revealed that hypoxia leads to the transcriptional activation of a large number of genes; these include the genes for erythropoietin, tyrosine hydroxylase, adenylate kinase-3, heme oxygenase, nitric oxide synthase and endothelin-1 (Ratcliffe et al., 1998). Hypoxia also leads to the transcriptional activation of genes for proteins involved in glycolysis, glucose transport, iron transport, angiogenesis (i.e. platelet derived growth factor and vescicular endothelial growth factor). The induction of these genes by hypoxia is mediated by hypoxia inducible factor 1 (HIF-1). This transcription factor is a heterodimer composed of a 120-kDa HIF-1α subunit and a 91–94-kDa HIF-1β subunit (Semenza and Wang, 1992; Wang and Semenza, 1993, 1995). Whereas the HIF-1α subunit is unique to HIF-1 the β subunit is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1 activates the expression of its target genes during hypoxia by binding to enhancer elements in their 5′ (e.g. VEGF gene) or 3′ flanking (e.g. EPO gene) regions. The results of studies that have measured the levels and stability of α and β subunits in cells grown at different oxygen tensions suggest that the α subunit is involved in regulating HIF-1 activity (Wang and Semenza, 1995; Huang et al., 1996). Recent studies have suggested that HIF-1 may not act alone to activate hypoxic genes; at least two other transcription factors, p300 and HNF-4, appear to be involved in the hypoxic induction of the Epo gene (Bunn et al., 1998). Similarly, additional *cis* elements and *trans* factors appear to be necessary for the maximal hypoxic induction of the LDH-A gene (Firth et al., 1995).

From the above, it is clear that the picture concerning the transcriptional machinery involved in oxygen-regulated expression of both mammalian and yeast genes is still incomplete. Unclear is how many other factors are involved in the oxygen-regulated expression of these genes, what role mediators and TAFS (i.e. coactivators) play, and whether mammalian cells have functional ho-

mologues of Hap1p or Rox1p. Also unclear is whether the redox-dependent assembly and DNA binding ability of NF-Y (Nakshatri et al., 1996), the mammalian homologue of yeast Hap2/3/4/5p, functions in oxygen-regulated expression of aerobic genes in mammals. Finally, although yeast cells lack HIF-1 it is not known if they have a protein homologue that functions like HIF-1 in activating the expression of hypoxic genes as cells experience low oxygen tensions. In view of these uncertainties it is not yet possible to say how similar oxygen-regulated transcription is in yeast and mammals.

5.2. Oxygen sensing and signal transduction

As discussed above, the dose–response curves relating steady state mRNA levels to oxygen concentration for aerobic genes support the hypothesis that heme is involved in at least two different ways in oxygen sensing in yeast. Moreover, the induction of a subset of hypoxic genes is inhibited by carbon monoxide, cyanide, respiratory inhibitors, and respiration-deficient mutations (Kwast et al., 1999), implicating the respiratory chain and cytochrome *c* oxidase in oxygen sensing and signal transduction for at least some hypoxic yeast genes.

Heme is also involved in oxygen sensing and signal transduction in higher eucaryotes. In mammals, it has been proposed that heme functions as a redox-sensitive component of a hemoprotein oxygen sensor (Model 2), during the hypoxic induction of several genes. This is supported by two types of findings (Bunn and Poyton, 1996). First, a number of hypoxic genes are induced not only by hypoxia but also by iron chelators and transition metals (e.g, cobalt and nickel), which are capable of compromising the ability of hemoproteins to bind oxygen. Second, carbon monoxide blocks the induction of these genes. These findings provide strong, but indirect, evidence that oxygen sensing in mammals involves a hemoprotein that binds oxygen. When the iron atom in heme is replaced by nickel or cobalt in this oxygen sensor the hypoxic state is mimicked (Goldberg et al., 1988). So far, two possible hemoprotein oxygen sensors have been identified. The first is a multisubunit plasma membrane bound cytochrome b NAD(P)H oxidase discovered by Acker and colleagues (Acker, 1994; Acker and Xue, 1995). This protein is capable of reducing oxygen to ROS (Fandrey et al., 1994; Gorlach et al., 1994), which have been proposed to function in a signal transduction pathway for oxygen sensing (Bunn and Poyton, 1996; Bunn et al., 1998). The second is cytochrome oxidase of the mitochondrial respiratory chain. Early evidence for the involvement of cytochrome *c* oxidase comes from spectral studies (Wilson et al., 1994; Lahiri et al., 1995, 1996) examining the influence of azide, cyanide, and CO on chemosensory discharge, primarily in carotid body cells (Acker and Xue, 1995; Bunn and Poyton, 1996). More recently, it has been found that part of the hypoxic induction of some genes is sensitive to cyanide and that the mitochondrial respiratory chain is required for the hypoxic induction of a number of genes, including Epo, and the genes for VEGF, aldolase, and phosphoglycerate kinase (Chandel et al., 1998). Although the above evidence support the conclusion that the redox state of a hemoprotein (Model 2) is involved in oxygen sensing in mammalian cells it is not yet known if heme concentration is also involved (Model 1).

6. Role of cytochrome *c* oxidase and the respiratory chain in the induction of hypoxic genes in yeast and mammals

One feature common to both yeast and mammalian oxygen sensing is the involvement of cytochrome *c* oxidase and the respiratory chain in the induction of some hypoxic genes (Chandel et al., 1998; Kwast et al., 1999). The mitochondrial respiratory chain and cytochrome *c* oxidase are well-suited for a role in oxygen sensing because they are consumers of most of the oxygen that is used by eucaryotic cells and because the flux of electrons through both the respiratory chain and cytochrome *c* oxidase is affected by oxygen concentration in several cell types, including yeast (Burke et al., 1998) and mammalian cells (Wilson et al., 1988; Rumsey et al., 1990; Gnaigger et al., 1995; Verkhovsky et al., 1996). Moreover, both

the apparent K_m for oxygen binding to cytochrome *c* oxidase and the rate of electron transfer from heme a to a_3 is affected by oxygen concentration (Verkhovsky et al., 1996). Currently, it is not clear how cytochrome *c* oxidase may 'sense' oxygen as cells are shifted into hypoxic or anoxic conditions, what signal is released from the mitochondrion during this process, and what pathway is involved in transducing this signal into an effect on nuclear gene expression. There are several possibilities. First, as noted by Wilson et al. (1988), mitochondrial oxidative phosphorylation is dependent on oxygen concentration; hence, mitochondria can convert information concerning cytosolic oxygen concentration into a metabolic signal (e.g. [ATP]/[ADP] [Pi], NAD(P)H/NAD(P)). This, in turn, can affect virtually every aspect of cell function, including the phosphorylation of transcription factors or other members of a signal transduction pathway. This model is intriguing because the threshold for expression of hypoxic yeast genes is low, 0.25 μM O_2 (Burke et al., 1997, 1998; Kwast et al., unpublished), and approximates the apparent K_m for oxygen binding to yeast cytochrome *c* oxidase (Poyton et al., unpublished). However, respiratory inhibitors and mutations affect expression of *OLE1* and *CYC7* under strictly anoxic conditions in which the respiratory chain is already inhibited by oxygen deprivation. These findings argue against a signalling pathway that involves global changes in cellular energy charge or redox but do not preclude a pathway linked to the respiratory chain itself. A second possibility, proposed for mammalian cells (Duranteau et al., 1998), is that the decrease in the V_{max} of cytochrome *c* oxidase in response to hypoxia (Chandel et al., 1996) increases the reduction state of mitochondrial electron carriers that are upstream of cytochrome *c* oxidase and, subsequently, the generation of reactive oxygen species (ROS) (Chandel et al., 1998; Duranteau et al., 1998). These ROS, which have been implicated in the crosstalk between the mitochondrion and nucleus (Poyton and McEwen, 1996), would then function to activate members of signal transduction pathways (e.g. kinases, nuclear transcriptions factors, etc.). It seems unlikely that this type of signaling is involved in the anoxic-induction of *OLE1* and *CYC7* in yeast because cyanide blocks their induction when added to anaerobic cells, in which ROS should be absent. This type of experiment has not yet been done with mammalian cells; consequently, the possiblility that some other factor(s) (see below) besides ROS are involved in mammals has not yet been ruled out. A third possibility is that a protein involved in a signal–transduction pathway serves as an electron acceptor or donor for cytochrome *c* oxidase, and that this protein is released under hypoxic or anoxic conditions directly into the cytosol, where it activates or inactivates transcription factors that regulate the expression of hypoxic genes. Precedent for this sort of pathway comes from the fact that mitochondria export proteins and peptides (Poyton et al., 1996), and that cytochrome *c*, which is immediately upstream of cytochrome *c* oxidase in the respiratory chain, is easily released from mitochondria under certain conditions (e.g. apoptosis) (Liu et al., 1996; Kluck et al., 1997). A fourth possibility is that the functional integrity of cytochrome *c* oxidase or other components of the respiratory chain is compromised as cells are shifted from aerobic into anaerobic conditions, and that respiratory chain subunits or subcomplexes are released from the mitochondrion and serve directly as 'signals' of hypoxia. It is well known that there is a coordinate loss of cytochromes after yeast cells are shifted from aerobic to anoxic conditions (Rep and Grivell, 1996) but it is not known how fast this occurs and whether it is fast enough to explain the kinetics of induction of hypoxic genes.

These possibilities are not mutually exclusive. Indeed, it is possible that more than one of them are at work. For example, disassembly cytochrome *c* oxidase resulting from a shift to hypoxic conditions, may lead to the release of subunits or subcomplexes into the cytosol and the leakage of reactive oxygen intermediates.

7. Future prospectives

During the past few years a great deal of progress has been made in understanding oxygen-reg-

ulated gene expression in yeast and mammals, organisms that define the opposite ends of the eucaryotic spectrum. But clearly, the picture for oxygen sensing and signal transduction in eucaryotes is incomplete. Still unclear is what cells sense as they experience a change in P_{O_2}, how 'oxygen' sensors sense P_{O_2}, how these sensors transmit the sensation to a signaling pathway(s) that results in the up- or down-regulation of genes, and whether there are universal and/or unique molecular mechanisms for responding to changes in oxygen concentration in different eucaryotes.

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RESPIRATION PHYSIOLOGY

Carotid body glomus cells: chemical secretion and transmission (modulation?) across cell-nerve ending junctions

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Abstract

Glomus cells of the carotid body contain and secrete chemicals during 'natural' stimulation (hypoxia, hypercapnia and acidity), thus, the birth of the 'transmitter hypothesis of chemoreception'. Released chemicals would cross the synaptic cleft between glomus cells and carotid nerve terminals to depolarize the nerve ending membrane during excitation and hyperpolarize the membrane during inhibition. The main problem with this hypothesis is that specific synaptic blockers modify but do not block the effects of natural stimulation, while blocking the effects of the putative transmitters. It is proposed in this review that the secretion of chemicals is modulated by changes in electric coupling between glomus cells and that glomus cell-nerve ending transmission is not blocked by specific blockers for two reasons. One is that multiple transmitters are released. The other, and more the likely explanation, is that there are electric connections between these elements allowing the flow of currents that are unaffected by the blockers.

Keywords: Carotid body, chemical transduction, gap junctions; Control of breathing, hypoxia, carotid body; Gap junctions, carotid body; Hypoxia, carotid body, signal transduction

The carotid body is unquestionably a polymodal receptor. The frequency of carotid nerve sensory discharges increases during hypoxia, hypercapnia, acidity, high temperature, hyperosmolarity and applications of a variety of drugs. Discharges decrease during hyperoxia, hypocapnia, cold and hyposmolarity (Eyzaguirre et al., 1983; Eyzaguirre and Zapata, 1984; Fidone and González, 1986; González et al., 1994; Zapata, 1997a). This lack of specificity has made it very difficult to study the mechanisms leading to the onset of the sensory discharge, thus, the birth over the years of many theories or hypotheses (depend-

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ing on your taste) of chemoreception. In this chapter, we will deal with one, the 'transmitter hypothesis', which is probably the most commonly accepted nowadays. However, as shown below, there are some holes in this idea. However, these problems are not unique in carotid body studies. This little 'bloody milligram' has become irritatingly elusive when trying to understand the mechanisms of action of different agents.

The carotid body fine structure is complex. The 'sensory unit' is formed by one carotid nerve fiber that branches extensively to innervate clusters of glomus (type I) cells surrounded by processes of sustentacular (type II) cells. Many glomus cells are in close apposition with terminals of carotid nerve fibers, forming a sensory synapse where the respective membranes are separated by a synaptic cleft 20 nm wide. The whole synaptic complex is enveloped by processes of the sustentacular cells. Glomus cells have a distinct appearance since they possess dark-core vesicles (similar to those in the adrenal medulla). Also, there are clear-core vesicles in the cells and in the nerve terminals. McDonald (1981) has shown that these synapses are morphologically polarized from cell to ending, from ending to cell and others are reciprocal. Sometimes, the three types of polarization have been found in a single junction. Furthermore, there are gap junctions between glomus cells, between glomus and sustentacular cells and between glomus cells and nerve terminals (within the chemical synapse). For details of carotid body morphology, see McDonald (1981), Verna (1997).

1. Basis for the transmitter hypothesis of chemoreception

There is no doubt that glomus cells of the carotid body contain several chemicals (see below). Also, it has been well established that catecholamines and acetylcholine (ACh) can be recovered during carotid body superfusion in vitro. The recovered amounts increase during stimulation by hypoxia, hypercapnia and acidity. These observations indicate that the carotid body is continuously releasing substances into its profuse circulation although systemic effects of this release have not been described. However, the most important aspect of chemical release is that it influences the pre and postsynaptic elements of the 'chemical' synapses occurring between the glomus cells and the carotid nerve terminals. In this context, it has been suggested that the released chemicals cross the synaptic cleft to depolarize the nerve ending membrane (postsynaptic in this case) if the chemical excites the nerve. If the substance inhibits the sensory discharge we should expect hyperpolarization of the nerve terminal membrane. These are the foundations of the so called 'transmitter hypothesis of chemoreception'. It should be said, however, that this hypothesis is one of many that have been formulated trying to explain the origin of sensory impulses in the carotid nerve.

Carotid body glomus cells meet many of the requirements expected of presynaptic structures involved in chemical transmission: (1) they possess the enzymatic machinery to produce these chemicals; (2) different stimuli release these substances; (3) exogenous applications of the putative transmitters change the carotid nerve sensory discharge frequency; and (4) there is direct and indirect evidence that excitatory chemicals depolarize the nerve endings whereas inhibitory agents hyperpolarize them. Therefore, we have a situation in which the glomus cells produce and secrete several chemical substances that, if exogenously applied, activate or inhibit the nerve terminals. However, the main stumbling block to accept chemical transmission, as responsible for the onset and frequency changes of the sensory discharge, is the relative ineffectiveness of specific synaptic blockers. Thus, substances released from glomus cells may only be 'modulators' of the sensory discharge, or their transmitter action is either hidden or impaired by other factors. It is unreasonable to assume that these chemicals have no effect whatsoever in the generation of sensory impulses. Carotid body stimulation releases enough quantities of them, judging from the amounts collected in vitro, and even a small concentration should be very effective when leaking into the synaptic spaces. Also, as just mentioned, exogenous administrations are effective on the nerve terminals. What follows is an attempt to find an explanation to this puzzle.

2. Glomus cells contain and release multiple substances

One of the principal problems facing the transmitter hypothesis is that the presumed presynaptic elements of the glomus cell-nerve ending junctions contain numerous chemicals that are potential transmitters. Thus, if more than one (or several) are released during receptor activation, it is virtually impossible to block transmission using a specific synaptic blocker. One would need to use a cocktail of blockers, a procedure that would horrify a respectable pharmacologist.

As mentioned before, the carotid body contains catecholamines, especially DA, and ACh. These two substances are not just stored in the cells of the organ but are produced in them. Numerous studies have shown the presence of the synthesizing enzyme for catecholamines, tyrosine hydroxylase (TH) in glomus cells. Similarly, acetylcholine transferase, the synthesizer for ACh has also been found in these cells. To make matters worse, 5-hydroxytryptamine, met and leu-enkephalins, neuropeptide Y, calcitonin-gene-related peptide (CGRP), endothelins, galanin, cholecystokinin, and atrial natriuretic peptide (ANP) also have been found in glomus cells. Other substances present in the carotid body such as vasoactive intestinal peptide (VIP) and nitric oxide (NO) are mainly located in the carotid nerve fibers. They may play a role in eliciting the sensory discharge, perhaps by acting on the glomus cells. This appears to be the case with NO that changes the cGMP levels in these cells. Substance P has been found in the nerve fibers and glomus cells of the carotid body (González et al., 1997; Verna, 1997; Zapata, 1997b).

Catecholamines (especially DA) and ACh are known to be released during natural carotid body stimulation (hypoxia, hypercapnia, acidity) and change the sensory discharge when exogenously applied. The assumption is that these substances cross the synaptic cleft and change the polarization of the nerve ending membrane. For this process to occur, it is necessary to have specific receptors for these agents in the nerve ending membrane. The following are some problems.

Concerning ACh, nicotinic and muscarinic receptors (respectively identified by alpha-bungarotoxin and quinoclinidyl benzilate radioligands) have been found in the glomus cells but not in the nerve endings. However, alpha-bungarotoxin radioligands miss about one half of nicotinic receptors in glomus cells, and nicotinic cholinergic receptors in nerve endings may be insensitive to this venom. In fact, Ishizawa et al. (1996) using monoclonal antibodies have found nicotinic receptors in cat glomus cells and petrosal ganglion neurons. The latter finding probably indicate that cholinergic receptors occur in carotid nerve endings. With regard to catecholamines, dopaminergic D_1 receptors have been identified in glomus cells and in petrosal ganglion neurons, presumably innervating the carotid body, suggesting that these receptors occur both in the cells and carotid nerve terminals (González et al., 1997).

There is no direct evidence demonstrating the release of the other substances but they may affect the sensory discharge, judging from results obtained with pharmacological applications. If they are effective physiologically, some release toward the nerve terminals is necessary, unless they act indirectly by acting on specific receptors in the glomus cell membrane.

3. Pharmacological observations related to transmitter action

A substance can be thought of as a transmitter if: (a) it mimics the effects of presynaptic stimulation when exogenously applied; and (b) a specific synaptic blocker eliminates the postsynaptic effects of presynaptic stimulation and of the presumed transmitter. As extensively reported elsewhere, neither ACh nor dopamine (DA, the most investigated agents) rigorously fit these criteria. There are species differences in the effects of these drugs, and in vivo and in vitro experiments not always yield qualitatively identical results.

3.1. Acetylcholine (ACh)

Exogenous applications of ACh vigorously excite carotid nerve fibers resulting in an increased

sensory discharge. This is true in all species except rabbits, where ACh depresses the carotid nerve discharge in vivo and in vitro. It is thought that excitation by ACh is due to activation of nicotinic receptors, and depression by an effect on muscarinic receptors. This concept is clearly shown in cat and rabbit carotid body chemoreceptors. In both species, applications of nicotine excite the receptors whereas muscarinic agents, such as pilocarpine and bethanechol, are ineffective in the cat and depress the discharge in the rabbit (McQueen, 1977; Docherty and McQueen, 1979; Monti-Bloch and Eyzaguirre, 1980). These findings would suggest that ACh, endogenously contained in and released from glomus cells, is an excitatory transmitter operating between the glomus cells and the nerve terminals. However, the following is disturbing.

3.2. Cholinergic blockers

Old observations showed that applications of TEA (a cholinergic ganglionic blocker and depressant of voltage-gated K^+ channels) blocked the effects of ACh on chemoreflex excitation. However, the drug had little effect when these reflexes were evoked by 'natural' stimuli such as hypoxia and hypercapnia (Moe et al., 1948; Anichkov and Belen'kii, 1963). Later observations extended these findings by including other cholinergic blockers such as curare, hexamethonium (C_6), mecamylamine and atropine. These experiments, conducted in vivo and in vitro, used recordings from the carotid nerve instead of ventilatory reflexes. In most cases, the nicotinic blockers (curare, C_6, mecamylamine) blocked the effects of exogenous ACh but only increased the threshold of 'natural' excitation. Rarely, the agents blocked 'natural' excitatory effects. Atropine, was generally ineffective in blocking either form of stimulation (Eyzaguirre and Zapata, 1968b; Nishi and Eyzaguirre, 1970; McQueen, 1977). More recently, Fitzgerald et al. (1997) have found that both mecamylamine and atropine depress the chemoreceptor response to hypoxia. But again, there is no block since they found only a reduced response.

The earlier findings led Douglas (1954) to write "the argument [cholinergic hypothesis of chemoreception] stands, as it were, like an old fashioned stool upon three legs". He continues to say that the first leg is missing since ACh had not been recovered from the carotid body. We know now (see above) that ACh has been recovered from this organ during natural stimulation. Douglas contended that the second leg of the stool was not firmly attached because there was uncertainty about the effects of anticholinesterases on the sensory discharge. However, later evidence has shown that anticholinesterase drugs clearly increase the sensory discharge. He continues "What now of the third [leg]?". He argues that the third leg is pretty hollow because his own experiments showed that C_6 abolished the response to injected ACh without affecting the response to oxygen lack. Consequently, his conclusions were that the cholinergic hypothesis of chemoreception, initially advocated by von Euler et al. (1939) stood like a three-legged stool with 'one leg hollow, one wobbly and one missing'. This colorful and amusing allegory has been hard to discard even with a wealth of later evidence as indicated in the previous paragraph. However, when Douglas wrote these comments, all analogies with other cholinergic synapses were invariably directed toward the better known neuromuscular junction and sympathetic ganglion. Then and now, the neuromuscular junction is an exception where ACh is the exclusive transmitter. At the time, it was thought that ACh also was the transmitter in sympathetic ganglia. There was practically no information about the complexities of synapses in the central nervous system, in sympathetic ganglia and peripheral synapses in the autonomic nervous system. At that time, Dale's Law reigned supreme. We know now that many synapses have multiple transmitters and that second messengers play an important role in synaptic transmission. As shown above and below, more recent evidence suggests that the glomus cell-nerve ending junction is very complex since we have multiple transmitters, second messengers and, possibly, electric connections between glomus cells and nerve terminals. Therefore, the three legged stool may, after all, have three relatively firmly attached and semi-solid legs.

3.3. Dopamine (DA)

Exogenous administrations of DA block or depress the sensory discharge in all species (from man to mouse) studied in vivo. The exceptions occur in vitro where DA superfusion increases the discharge of the carotid nerve in the rabbit and also in the cat when DA doses are high (Zapata, 1975; Monti-Bloch and Eyzaguirre, 1980). These observations have provoked some interesting thoughts regarding the role of DA as a sensory transmitter. At first glance, and following accepted norms of transmitter identification, one would immediately think that DA is an inhibitory transmitter. However, González et al. (1997) reviewing their many years of superb experimentation on the subject suggest that DA is excitatory with the caveat that they are not absolutely certain about this point. They have found the presence of DA D_1 receptors in the carotid body vasculature and D_2 receptors in the glomus cells and petrosal sensory neurons (presumably associated with the carotid nerve terminals). Also, they mention that low doses of the drug inhibit the sensory discharge whereas large doses excite the receptors. The question is whether D_2 receptors (principally associated with inhibiting adenylate cyclase and phosphatidylinositol turnover) are involved in receptor excitation or inhibition. From a functional point of view it may be better at present to be a bit vague. Zapata (1997b) has proposed the presence of excitatory and inhibitory receptors (DA_e and DA_i) in the glomus cell-nerve ending complex, as proposed for other tissues. Following this concept, one would expect inhibition by DA when DA_i receptors predominate and excitation when there is predominance of DA_e receptors.

As catalogued by Zapata (1997b), DA agonists such as apomorphine and amantadine mimic the inhibitory effects of DA whereas DA antagonists (butyrophenones, phenothiazines, thioxantenes, ergoloids, benzamides and aralkylpiperidines) block the inhibitory effects of DA. The excitatory effects of DA on rabbit carotid bodies in vitro are not affected by blockers such as haloperidol and butaclamol.

3.4. Other possible transmitters

Other substances, contained in the carotid body glomus cells and nerves, have been studied less thoroughly from a pharmacological point of view.

(a) Substance P. Intracarotid administration of this peptide in the cat elicited an initial increase followed by decrease of the sensory discharge and the effects of NaCN were potentiated (McQueen, 1980). Prabhakar et al. (1990) have reported dose-dependent chemoreceptor excitation in the cat by intracarotid injections of Substance P. Furthermore, SP antagonists blocked the carotid body chemoreceptors response to hypoxia. In vitro, however, these effects of Substance P were less clear. The peptide increased or decreased the sensory discharge of the cat carotid nerve in almost equal proportions, excitation being more evident with larger doses. Substance P significantly depressed the effects of hypoxia, but those of hypercapnia and acidity were less clearly affected, Substance P tended to increase the effects of ACh (excitation) and of DA (inhibition) as shown by Monti-Bloch and Eyzaguirre (1985).

(b) Enkephalins. Vascular delivery of met-enkephalin and leu-enkephalin in the cat reduced the basal discharge and depressed the effects of injected ACh and NaCN (McQueen and Ribeiro, 1980). Similar effects have been observed in vitro during superfusion with met-enkephalin 10^{-8}–10^{-5} M. Also in vitro, met-enkephalin clearly depressed the effects of hypoxic hypoxia and hypercapnia but not those induced by acidity. All effects of enkephalins were blocked by previous administration of naloxone (Monti-Bloch and Eyzaguirre, 1985). Other studies have indicated that the effects of the enkephalins are produced through action on delta opioid receptors and that enkephalins may act by restraining the excitatory effects of hypoxia (Zapata, 1997b).

4. Glomus cells have autoreceptors for their own transmitters

There is considerable evidence that glomus cells react to the substances released from them. Ligand and monoclonal antibody studies have shown

that cat and rabbit carotid body glomus cells possess nicotinic and muscarinic receptors. Nicotinic receptors predominate in the cat whereas muscarinic receptors have a predominance in rabbit cells (Ishizawa et al., 1996; González et al., 1997). These findings are supported by physiological observations. We have found that ACh applied to intact and sliced carotid bodies depolarizes most glomus cells of the cat, rabbit and mouse. Applications of nicotine, pilocarpine or bethanechol had similar effects. The responses obtained by nicotine were depressed by alpha-bungarotoxin and curare whereas those induced by pilocarpine and bethanechol were depressed by atropine. However, no significant species differences were seen during administration of nicotine or bethanechol (Eyzaguirre and Monti-Bloch, 1982; Eyzaguirre et al., 1990). Thus, cholinergic receptors, other than those identified by ligand studies, probably occur in glomus cells. Recent studies have shown that rat glomus cells also possess nicotinic and muscarinic receptors. Their activation increases intracellular calcium (Dasso et al., 1997).

The observations just described make it very likely that released ACh, bathing the cells from which it was released, will in turn affect their membranes. This circuit should form a positive or negative feedback affecting the release of the same or another transmitter. In fact, Gomez-Niño et al. (1990) have shown that nicotinic agonists release catecholamines (especially norepinephrine) from rabbit glomus cells.

Concerning DA, Goldman and Eyzaguirre (1984) have shown that applications of DA in vitro to rabbit glomus cells depolarized more than 75% of them whereas the rest were hyperpolarized. These effects depended on the initial E_m. Cells with lower resting potentials (~ -22 mV) were depolarized while those with more negative resting E_ms (~ -37 mV) were hyperpolarized. DA induced the largest depolarizations when the E_m was artificially set at 0 mV. The E_m changes induced by DA (negative and positive) were produced by increases in membrane resistance.

These experiments suggest that DA leaking out of the cells is bound to affect its own release. The increased membrane resistance shows that pathways across the cell membrane are obstructed. This effect appears to be parallel to the reported feedback inhibition of DA release by this substance. In fact, application of DA D_2 receptor agonists inhibit DA release. One wonders, therefore, if exogenous DA partially blocks vesicular extrusion of dopaminergic vesicles. Unfortunately, there are no membrane capacitance studies during DA release. Such measurements would provide an accurate test of this possibility.

Thus, we can be fairly certain that released DA acts on its own receptors in the glomus cells to decrease or inhibit further release. Similarly, in all probability, released ACh also acts on its own receptors. We do not know, however, if this feedback is positive or negative because there are very few studies on ACh release from glomus cells. Furthermore, ACh also acts on DA receptors facilitating DA release.

5. A phenomenon accompanying secretion. possible role of intercellular coupling

What follows is not an attempt to explain or describe the intimate mechanisms responsible for secretion in glomus cells. Simply, the purpose of this section is to find a physiological meaning to intercellular coupling that we have amply described in recent years (Monti-Bloch et al., 1993; Eyzaguirre and Abudara, 1995, 1996; Abudara and Eyzaguirre, 1996, 1998).

Most cells in the body are dye- and electrically coupled with the exception of skeletal muscle fibers and many neurons in the central nervous system. A notable example, pertinent to this discussion, is given by the secretory cells of exocrine and endocrine glands. When an exocrine organ is stimulated, by a secretagogue for instance, secretion toward the duct is accompanied by uncoupling (or reduced coupling) of the secreting cells. In some endocrine organs, such as the endocrine pancreas, the opposite occurs, i.e. during secretion toward the blood capillaries, the endocrine cells undergo tighter coupling (Bennett and Spray, 1985).

Coupled cells show two distinct characteristics. One is that a dye injected into one cell spreads to

neighboring cells (Baron and Eyzaguirre, 1977). The other is that injected currents also spread, although attenuated, to the neighbors. This is possible when gap junctions occur between the cells. The term gap junction is a misnomer adopted when in early work with the EM researchers observed that the membranes of two adjoining cells became very close for small stretches. We know now that each gap junction is cribriform, being perforated by hundreds or thousands of intercellular channels that connect the cytosols of adjoining cells. The pores of the channels are surrounded by six protein formations on each side (hexameric structure) leading to a dodecameric structure on both sides of the membranes. It has been suggested that the intercellular channels open and close like the shutter of a photographic camera (Hille, 1992). When we talk about tight coupling, the intercellular channels are open, allowing flow of materials (even small molecules) and currents between the cells. When the channels close (or become less open), the flow of materials and currents are impeded and we talk about cell uncoupling. Consequently, we can test coupling or its changes during different procedures (stimulation for example) by observing changes in the flow of dyes or the ease of intercellular (junctional) current flow. The latter (I_j) can be measured directly when two adjoining cells are voltage-clamped or indirectly by measuring the voltage drop across the junctions (V_j) under current clamping. The latter method has the advantage that one can study the effects of membrane potential changes during coupling.

With this background, we can discuss some of our results concerning intercellular coupling and the possible implications on glomus cell secretion. It must be said at the outset that there are no studies simultaneously studying both phenomena. We have studied intercellular coupling and others have studied chemical release from glomus cells. What follows is a brief review of results obtained during our studies on coupling. We have deliberately selected experiments using current clamping on isolated but whole carotid bodies for two reasons: (1) cultured cells provide excellent preparations (which we have extensively used) but it is uncertain whether cultures provide a situation resembling the normal functioning of the organ; and (2) voltage clamping (which we have also used) prevents the cell membrane to depolarize or hyperpolarize during stimulation thus impeding possible effects of voltage-gated channels. However, in this discussion we will use a voltage-clamp experiment to compare results obtained with current clamping.

Fig. 1(A,B) show an experiment in which two adjoining glomus cells of the carotid body in vitro (Cells 1 and 2) were simultaneously impaled with microelectrodes connected to independent amplifiers for current injections and voltage recordings. In the control period, the organ was superfused with physiological saline equilibrated with air. (A) shows that Cell 1 had a resting potential (E_m) at ~ -35 mV whereas the E_m of Cell 2 was at ~ -30 mV. At ~ 15 s, superfusion with saline containing $Na_2S_2O_4$ 1 mM (Na-DTN) started. The reducing agent decreased PO_2 from ~ 120 to 10 Torr (not illustrated) and both cells depolarized. Their E_ms reached values close to 0 mV.

The inset in B shows the stimulating and recording conditions used to establish the degree on intercellular coupling. Coupled Cells 1 an 2 are stimulated and recorded from. Positive pulses delivered to Cell 1 produced a relatively large depolarization in this cell (V_1) that spreads to Cell 2 across junction channels (arrow to the right) eliciting a smaller voltage (E_2). Negative pulses delivered to Cell 2 elicited large negative voltages (V_2) in this cell, spreading to Cell 1 (arrow to the left) giving rise to smaller voltage drop (E_1). The ratios E/V are the coupling coefficients (K_c). We delivered positive pulses to one cell and negative ones to the other for ease of identification in the traces, and there were no significant differences in the effects of either pulse polarity.

The traces in B show the fate of K_c during superfusion with $Na_2S_2O_4$. Both cells were tightly coupled. In Cell 1, K_c was ~ 0.75 (75% coupling) and in Cell 2, it was ~ 0.9 (90% coupling). During $Na_2S_2O_4$, coupling was reduced to $\sim 15\%$ (K_c at ~ 0.15).

An inspection of Fig. 1(A,B) shows that cell depolarization and decreased coupling occurred in opposite directions, and uncoupling appeared to be associated with depolarization. However, using

different stimuli (hypoxia, hypercapnia, acidity and applications of putative transmitter such as DA and ACh) not all cells depolarized. This phenomenon occurred in 70–80% of the cases whereas the rest hyperpolarized. Therefore, it was important to establish correlations between changes in E_m and changes in K_c. This analysis is presented in Fig. 1(C). In spite of the scatter of the data, there was a significant ($P < 0.006$) and negative correlation between ΔK_c and ΔE_m in 129 coupled cells. It shows that cell depolarization was more likely to be accompanied by a decrease in K_c or reduced intercellular coupling. This is important because glomus cell depolarization has been associated with glomus cell secretion (Weiss and Donnelly, 1996), and by extrapolation one would think, also with cell uncoupling. However, there are some problems with this reasoning.

Firstly, intercellular coupling is quite independent of the membrane potential. One has to displace the E_m by more than ± 40 mV to obtain significant changes in intercellular conductance (increases or decreases). One rarely sees changes in E_m of this magnitude during applications of different stimuli. Secondly, intracellularly applied pulses of either polarity decrease coupling, in proportion to their amplitude as shown in Fig. 1(D). This means that negative pulses depolarize the cell whereas positive pulses induce cell hyperpolarization. Thirdly, when both cells are voltage-

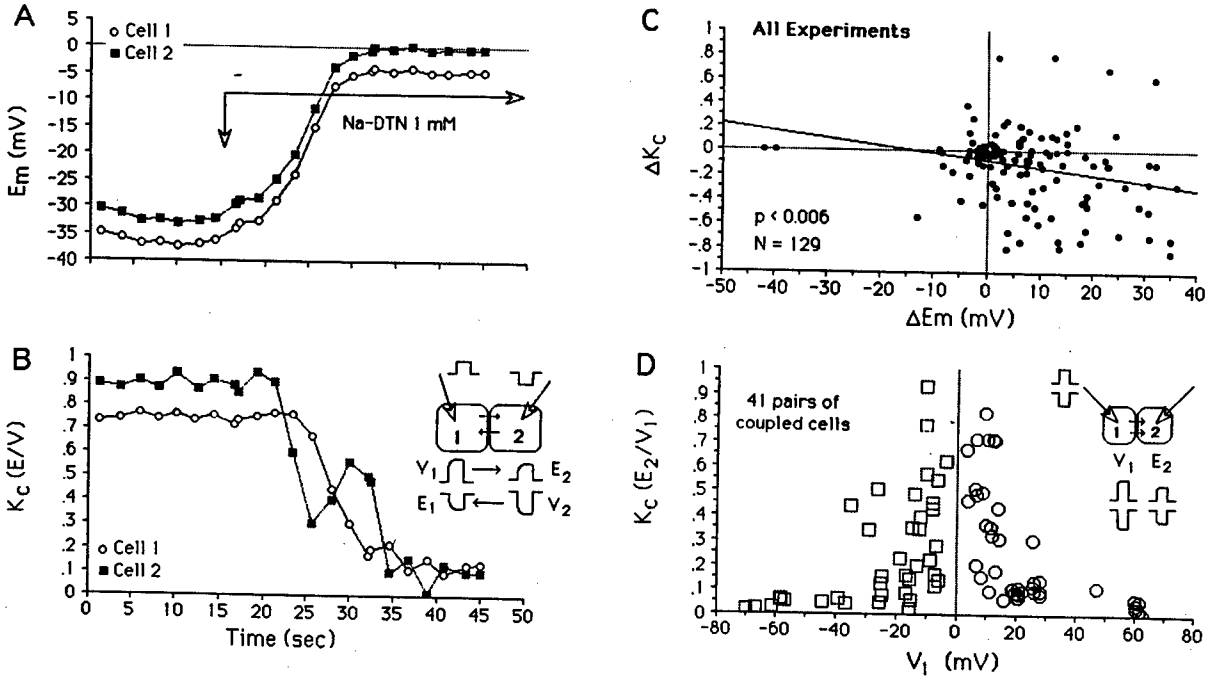


Fig. 1. Current clamping of coupled pair of glomus cells. Rat carotid body in vitro. (A–B) effects of $Na_2S_2O_4$ (Na-DTN). The inset in B shows the experimental conditions. Cells 1 and 2 are impaled with microelectrodes for voltage recordings and stimulation. A, superfusion with Na-DTN 1 mM at 15 sec markedly depolarizes Cell 1 (open circles) and Cell 2 (filled squares), from -30 and -35 mV to -5 and 0 mV. In (B), Cell 1 is stimulated with positive pulses that induce a large voltage drop across its membrane (V_1), which is transferred across intercellular junctions to Cell 2 (arrow to the right) and is recorded as E_2. Cell 2 is stimulated with negative pulses creating a negative voltage drop across its membrane (V_2), which is transferred across the junctions to Cell 1 (arrow to the left) and is recorded as E_1. (B) shows the progression of the coupling coefficients, $K_c = E/V$, before and during Na-DTN. At 20 sec, both cells begin to uncouple and K_c decreases from 0.7 and 0.9 to ~ 0.1 in both cells. (C) illustrates the significant ($P < 0.006$) correlation between changes in K_c (ΔK_c in the ordinate) and resting potentials (ΔE_m in the abscissa) in 129 coupled pairs subjected to different stimuli. (D) describes the effects of applying positive and negative pulses of different amplitudes on Cell 1 (V_1) and on coupled Cell 2 (E_2) as shown in the inset. K_c (abscissa) decreases almost equally when V_1 (ordinate) increases, regardless of V_1 polarity (Monti-Bloch et al., 1993).

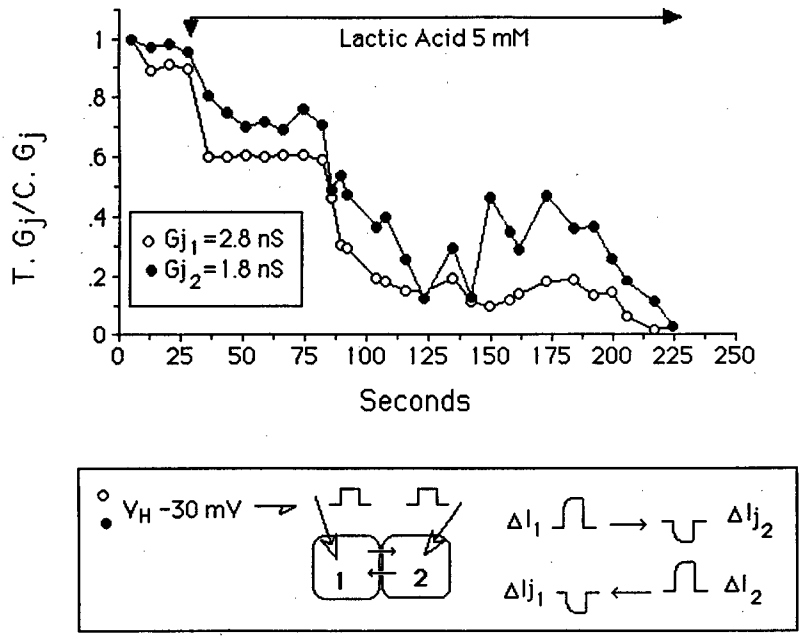


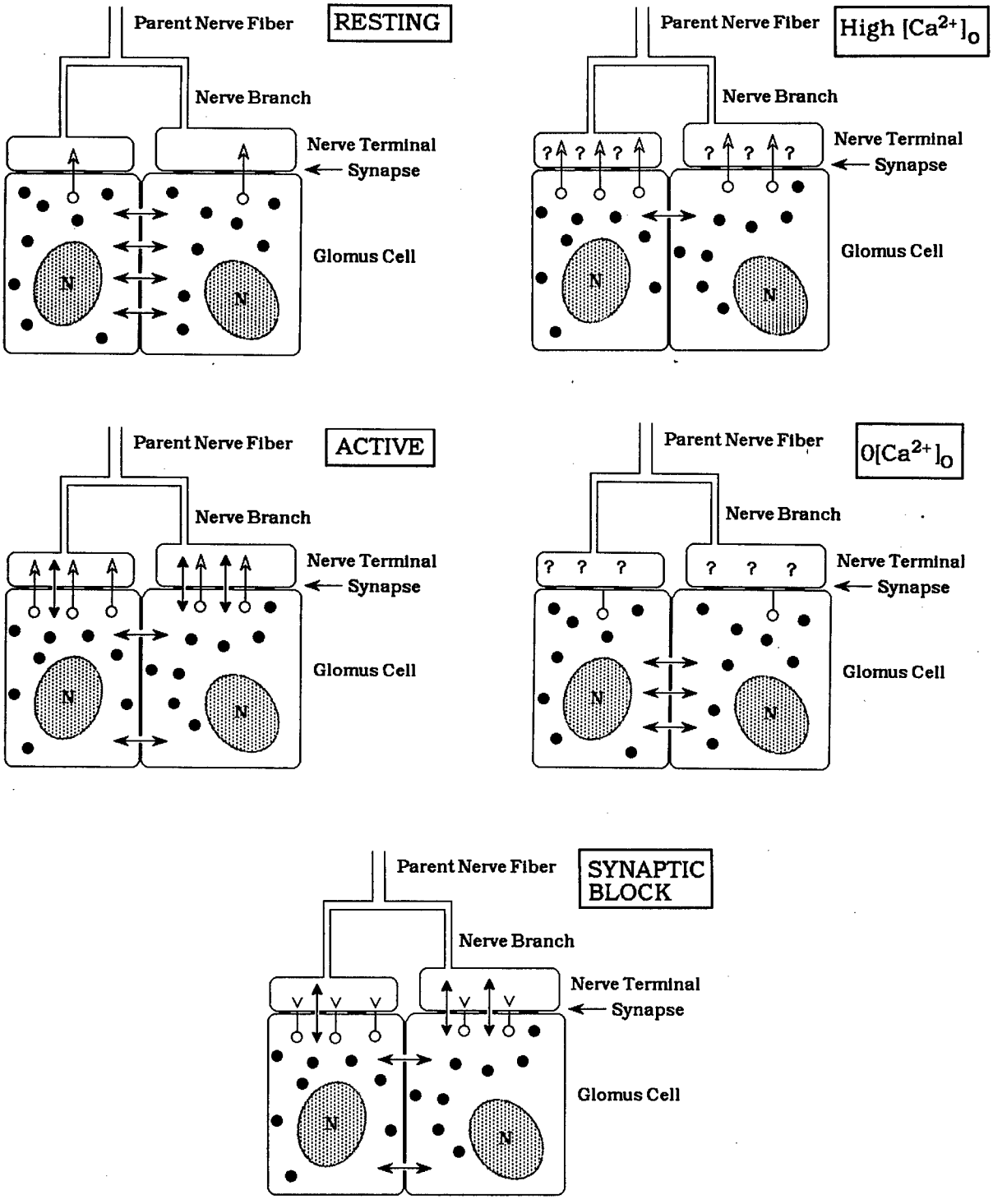
Fig. 2. Experiment designed to prevent voltage shifts. One pair of cultured rat glomus cells is voltage-clamped as shown in the inset. Both cells are voltage-clamped at −30 mV (value between their E_ms). Positive pulses shifted the clamping level toward 0 mV. This procedure elicited a brief positive current in Cell 1 (ΔI_1) that flowed into Cell 2 as an inward (junctional) current (ΔIj_2). The positive pulses applied to Cell 2 induced a brief positive current in this cell (ΔI_2) and a junctional current (ΔIj_1) that flowed into Cell 1. Current flows through the intercellular channels are depicted as arrows to the right or left, depending on the direction of current flow. The graph shows that control junctional macroconductances (G_j), calculated as I_j/V, were 2.8 nS for Cell 1→Cell 2 (open circles) and 1.8 nS for Cell 2→Cell 1 (filled circles). Superfusion with lactic acid 5 mM at 25 sec reduced intercellular conductances to 20–40% of the controls. Ordinate, $T \cdot G_j$ (test conductance)/$C \cdot G_j$ (control conductance). Abscissa, time in sec. (Data from Abudara and Eyzaguirre, 1998).

clamped as in Fig. 2, and the cell's E_ms are not allowed to be displaced, intracellular pulses clearly uncouple the cells. Consequently, glomus cell secretion seems to be dependent on membrane depolarization and, presumably, on intercellular uncoupling. However, changes in E_m and cell coupling are parallel but independent phenomena.

5.1. Theoretical discussion concerning a possible role of intercellular coupling on glomus cell secretion

McDonald (1981), using the EM, found gap junctions between rat glomus cells in ~3% of his observations. Our electrophysiological experiments have shown more (~30%) intercellular junctions between glomus cells in the same species. Abudara and Sáez (unpublished), using specific antibodies for connexin C-43 and examined with the light microscope, have seen frequent connecting junctions between rat glomus cells. These observations indicate that the EM (a magnificent tool for structural analyses) misses most junctions. This is not surprising since using the EM is like scanning a mural with a high power telescope. Electrophysiological experiments and immunocytochemical observations are much more accurate in providing the whole picture about intercellular junctions.

We propose that under normoxia and lack of stimulation, glomus cells are relatively well coupled (RESTING in Fig. 3) allowing flow of molecules and ions between the cells (horizontal double arrows). Release of transmitters toward the nerve endings (open vertical arrows linked to open circles) is minimal. During stimulation by


Parent Nerve Fiber
RESTING
Nerve Branch
Nerve Terminal
Synapse
Glomus Cell
N
Parent Nerve Fiber
High [Ca2+]o
Nerve Branch
Nerve Terminal
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Parent Nerve Fiber
ACTIVE
Nerve Branch
Nerve Terminal
Synapse
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SYNAPTIC BLOCK
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Fig. 3.

hypoxia, hypercapnia or acidity (ACTIVE in Fig. 3) some of the intercellular junctions close and there is more abundant release of transmitters toward the nerve terminals, across the glomus cell-nerve terminal junctions. This proposal is based on the fact that electrophysiological experiments have shown that most intercellular junctions close during the action of these stimuli (Monti-Bloch et al., 1993; Abudara and Eyzaguirre, 1998). Also, we know that there is release of transmitters such as DA and ACh during stimulation. When the secretory process is enhanced by increased extracellular calcium (High $[Ca^{2+}]_o$ in Fig. 3) intercellular coupling mainly decreases (Abudara and Eyzaguirre, 1996) and this is accompanied by increased transmitter release toward the nerve terminals. In this context it is important to emphasize that high $[Ca^{2+}]_o$ increases the basal discharge and the effects of natural stimuli. However if $[Ca^{2+}]_o$ is excessive, these effects are depressed (Eyzaguirre and Zapata, 1968a), which is not surprising since high $[Ca^{2+}]_o$ stabilizes excitable membranes preventing depolarization. On the other hand, when transmitter release is impaired by removing $[Ca^{2+}]_o$ (0 $[Ca^{2+}]_o$ in Fig. 3) most intercellular junctions open but there is little or no transmitter release toward the nerve endings. These assumptions, related to changes in $[Ca^{2+}]_o$, are based on physiological and biochemical observations. The presence of $0[Ca^{2+}]_o$ increases intercellular coupling (Abudara and Eyzaguirre, 1996) and drastically reduces the amounts of transmitter obtained during normoxia and during stimulations by hypoxia, hypercapnia and acidity. Additionally, $0[Ca^{2+}]_o$ reduces the basal chemoreceptor discharge and reverts to depression the excitatory effects of anoxia, cyanide and acidity (Eyzaguirre and Zapata, 1968a; Eyzaguirre and Nishi, 1976).

A corollary to this hypothesis is that glomus cells function as a push–pull pump. At rest, most glomus cells are coupled because numerous intercellular channels are open. During stimulation most (70–80%) glomus cells uncouple and release their contents toward the terminals. However, as said before, 20–30% of the cells undergo tighter coupling. We propose that these cells are 'recharging' by accumulating transmitters and not releasing them. If the stimulus is prolonged, the recharged cells will uncouple and release their contents whereas the previously uncoupled cells will begin to tighten intercellular coupling and recharge. In this fashion, the carotid body is capable of sustained activity, without exhausting its reserves, during intense and prolonged stimulation. These ideas fit well with what has been known for a long time concerning the carotid nerve discharge. During chronic or prolonged acute hypoxia, the carotid nerve is able to sustain a high frequency discharge for long periods. This would be impossible if a strong stimulus would activate all glomus cells at once and deplete their reserves in a short time (Eyzaguirre and Abudara, 1995, 1996).

Kondo and Iwasa (1996) have recently shown the presence of gap junctions, not only between glomus cells, as McDonald did, but also between glomus and sustentacular cells. These findings are

Fig. 3. Schematic diagram showing hypothetical transmission mechanisms in glomus cells. Paired and coupled glomus cells are interconnected by gap junctions that allow flow of molecules and ions between them (horizontal filled arrows). Both cells are innervated by branches of the carotid nerve (parent nerve branch) that divides into nerve branches. Nerve terminals from the latter make synaptic contact with the glomus cells. The synapse is depicted as being both chemical and electrical. RESTING, shows good coupling at rest between the cells and little flow of transmitters toward the nerve terminals (vertical open arrow linked to open circle). The glomus cell-nerve ending electric junctions are closed. ACTIVE, the intercellular junctions close and cells uncouple. At the same time, there is abundant flow of transmitters toward the nerve terminals through the chemical junctions. The electric junctions (within the chemical synapse) open permitting flow of ions between the nerve and the glomus cell (vertical and filled double arrows). HIGH $[Ca^{2+}]_o$ shows that the glomus cell uncouple and there is an increase in transmitter flow toward the nerve endings. $0[Ca^{2+}]_o$ indicates that intercellular coupling increases whereas there is little or no transmitter release toward the nerve. The functional situation of the glomus cell-nerve ending electric junctions during HIGH $[Ca^{2+}]_o$ and $0[Ca^{2+}]_o$ is unknown (??). SYNAPTIC BLOCK, shows that during natural stimulation and the influence of a specific synaptic blocker, there is glomus intercellular uncoupling, the chemical synapses are blocked (V) but the electric junctions remain open permitting electric transmission between glomus cells and nerve endings.

interesting but unusual. Glomus cells are neuron-like whereas sustentacular cells are glia-like (Kondo et al., 1982). Gap junctions have been found between neurons and occur frequently between glial cells. However, generally there are no gap connections between neurons and glia. An exception has been found in a coelenterate where gap junctions occur between receptor and enveloping cells. The morphological findings of Kondo and Iwasa (1996) support physiological observations where the behavior of cultured glomus cells is different when they are isolated and when their sustentacular envelope is preserved (Pang and Eyzaguirre, 1992, 1993). It indicates that secretion of chemicals by the glomus cells is bound to be affected by the sustentacular envelopes. Neuronal behavior is markedly influenced by surrounding glia, which is formed by metabolically and electrophysiologically active cells (Astion and Orkand, 1988; Attwell et al., 1991). Unfortunately, in the carotid body we know practically nothing about the behavior of sustentacular cells, and little about their effects on glomus cells.

6. Possible answers to the lack of effectiveness of synaptic blockers

6.1. Multiple transmitters and/or electric connections between glomus cells and nerve terminals

As mentioned above, multiple agents contained in glomus cells could act as synaptic transmitters in the chemical synapses connecting glomus cells and carotid nerve terminals. Of all these chemical substances, only ACh and DA have been recovered from effluents during carotid body stimulation. Thus, it is likely that a stimulus will release more than one transmitter making it very difficult for a specific synaptic blocker to be fully effective. Also, this has been the general experience in this type of experiment. However, we must take into consideration that the effects of applied ACh and catecholamines are usually antagonistic, as generally occurs in the peripheral autonomic nervous system. Synergism is rare and possibly restricted to the salivary glands. Consequently, if we block an excitatory transmitter (e.g. ACh), the inhibitory effects of DA should be more evident. This situation apparently does not happen. Conversely, if we block the inhibitory effects of DA, the excitatory effects of released ACh should be more pronounced. There are reports that administrations of haloperidol increases the basal discharge of chemoreceptor fibers (Zapata, 1997b). These pharmacological synergisms and antagonisms may occur, but we propose below an alternative (not necessarily exclusive) hypothesis. Essentially, synaptic blockers are ineffective because there are electric connections between glomus cells and nerve terminals.

Kondo and Iwasa (1996) occasionally found gap junctions interspersed in the chemical synapses between glomus cells and carotid nerve terminals. Again, their study employed the EM, which is an inadequate tool for general exploration of a tissue. We suspect that these junctions occur much more frequently, as with electric junctions between glomus cells. The validity or falsehood of this point will be established once immunocytochemical studies with antibodies specific for connexins are conducted. Consequently, the role of gap junctions in transmission between glomus cells and nerve terminals is, at present, an open question. Nevertheless, we propose that electric junctions play a crucial role in this transmission and that they are responsible (at least in part) for the lack of effectiveness of specific synaptic blockers.

We hypothesize the following. In a resting preparation (RESTING in Fig. 3), the electric junctions between glomus cells and nerve terminals are closed. Thus, there is no electric coupling under these conditions. During activation by natural stimuli, the electric cell→nerve junctions open, allowing flow of ions and materials between these structures (vertical and filled double arrows in ACTIVE in Fig. 3). This hypothetical situation would be the opposite of what occurs between glomus cells where the open junctions mostly close during activation. At present, it is difficult to speculate on what happens during changes in $[Ca^{2+}]_o$ (see ?? in High $[Ca^{2+}]_o$ and $0[Ca^{2+}]_o$ in Fig. 3). If high $[Ca^{2+}]_o$ uncouples, as between glomus cells, it would have no effect if the glomus

cell-nerve ending junctions are already closed. On the other hand, if 0[Ca^{2+}]$_o$ increases coupling it could explain the residual discharge and the effects of different stimuli under 0[Ca^{2+}]$_o$. In fact, when [Ca^{2+}]$_o$ is replaced with equimolar concentrations of Mg^{2+} the basal chemoreceptor discharge decreases and the increased discharge elicited by acid, anoxia and flow interruption reverted to depression (Eyzaguirre and Zapata, 1968a; Eyzaguirre and Nishi, 1976).

What is most important in this discussion is presented in SYNAPTIC BLOCK in Fig. 3. A specific synaptic blocker should block the effects of the released transmitter on the postsynaptic membrane of the nerve ending. However, if there are sufficient electric connections between glomus cells and the terminals, currents (ions) should still flow in that direction and transmission should not be blocked. There is no direct proof supporting this hypothesis. However, the bulk of available evidence, concerning the poor performance of cholinergic and dopaminergic blockers, is clearly in its favor.

7. Conclusions

It is clear that glomus cells contain and release more than one chemical substance. The main problem is whether we are in the presence of genuine transmission or 'modulation' as proposed by Zapata (1975). Classically, a transmitter is a substance produced in and released from the presynaptic element of a junction. A second condition is that applications of the putative transmitter mimics the effects of the natural stimulus. And third, a specific blocker for the substance should eliminate both the effects of the applied suspect and of natural stimulation. This classical concept was complicated later on by the recognition that second messengers modify the effects of primary transmitters.

In the chemical transmission hypothesis, glomus cells have been identified as the presynaptic elements of chemical junctions with carotid nerve endings. The nerve terminals apposed to them would be the postsynaptic structures. There is little doubt that glomus cells produce and release chemicals that could be transmitters (e.g. DA and ACh). When the released substances are exogenously applied their effects are species-consistent except in the rabbit. DA inhibits the sensory discharge at low doses but increases it at high concentrations. ACh, with all doses, increases the carotid nerve discharge frequency. DA blockers may increase the basal discharge frequency, but these blockers and those for ACh do not block the effects of natural stimuli. Both DA and ACh blockers eliminate the effects induced by exogenous applications of DA or ACh. Thus, it is tempting to dismiss them as transmitters and accept Zapata's idea that they are modulators, that is modifiers but not originators. On the other hand, they may be transmitters whose action is camouflaged by electric connections between glomus cells and nerve terminals.

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K^+ currents of glomus cells and chemosensory functions of carotid body

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Abstract

The mechanism by which the carotid body senses hypoxia is not resolved, but the glomus cell, a secretory cell apposed to the afferent nerve endings, is believed to play an essential role. It is proposed that hypoxia causes glomus cell depolarization, leading to activation of voltage-gated calcium influx and enhanced secretion of an excitatory transmitter. The initial step, hypoxia induced depolarization, may be mediated by several candidate K^+ channels which are sensitive to hypoxia, including: (1) a transient, voltage-dependent current; (2) a calcium and voltage dependent current; and (3) a non-voltage dependent, leak K^+ current. If these channels represent the initial step in the hypoxia transduction cascade then it would be expected that K^+ channel blocking agents would mimic the hypoxia response, leading to glomus cell secretion and increased nerve activity. This has been tested for the first two channels which are sensitive to classical K^+ channel blocking agents, and, in general, results have not borne out this prediction. At present, the pharmacology of the leak K^+ channel is not determined, and the experiment has not been undertaken. Thus, at present, hypoxic inhibition to a K^+ channel in the glomus cell may initiate chemotransduction but there are many unanswered questions, especially the failure of K^+ channel blocking agents to emulate the hypoxic response.

Keywords: Chemotransduction, carotid body; Control of breathing, carotid body; Glomus cell, depolarization, K^+ channels; Hypoxia, sensing, glomus cells

1. Peripheral chemoreceptors: overview and transduction schema

The carotid body is located at the bifurcation of the common carotid artery into the external and internal carotid arteries, and rapidly responds

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to a decrease in arterial oxygen tension or increase in blood acidity by increasing afferent activity (González et al., 1994). This, in turn, initiates a number of protective reflexes, including hyperventilation and arousal. The carotid body is innervated by a branch of the glossopharyngeal nerve, the sinus nerve, from which 200–600 axons (in the rat) branch and terminate in apposition to the 'glomus cell', a secretory cell containing large dense-cored vesicles and some clear-cored vesicles (McDonald and Mitchell, 1975). The glomus cell appears to be an essential element of hypoxia transduction, since: (1) cryo-destruction of the glomus cell or removal of the carotid body generally ablates any evidence of chemosensitivity on the sinus nerve neuroma (Verna et al., 1975; Ponte and Sadler, 1989); and (2) reestablishment of chemosensitivity following sinus nerve trans-section generally corresponds with the afferent nerve fibers reaching the carotid body (Zapata et al., 1977; Ponte and Sadler, 1989).

The mechanism of chemotransduction has remained elusive, but there is, what we may call, the standard model (Fig. 1). In this schema, hypoxia and hypercapnia lead to depolarization of the glomus cell resulting in activation of voltage-dependent calcium currents and an influx of calcium. This increase in intracellular calcium leads to secretion of an excitatory neurotransmitter from the glomus cell, afferent nerve depolarization and an increase in afferent nerve spiking activity. Each of these steps is controversial, perhaps none more-so then the initial step of glomus

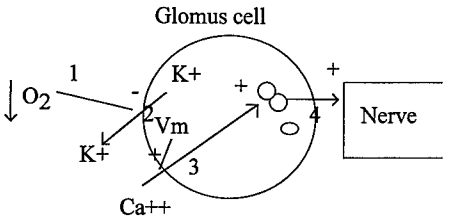

Fig. 1. Schematic diagram of a proposed schema for carotid body hypoxia transduction. Hypoxia inhibits an oxygen-sensitive K^+ current in the glomus cell (1), leading to depolarization (2) and influx of calcium through voltage-gated calcium channels (3). This leads to secretion of an excitatory neurotransmitter which depolarizes the afferent nerve endings (4) resulting in enhanced spiking activity.

cell depolarization by hypoxia and acidity. First reported in 1988 was the exciting observation that rabbit glomus cells have a K^+ current which is inhibited by hypoxia and which may potentially start the transduction cascade shown in Fig. 1 (López-Barneo et al., 1988). Subsequent work has shown that glomus cells may possess other types of oxygen-sensitive K^+ currents, but it remains unclear which, if any, of these oxygen sensitive K^+ currents is essential for organ function. In fact, initial observations suggested that K^+ ions and K^+ channels played little role in glomus cell function.

For a number of years, it was unclear whether K^+ ions participated in determining the resting potential of glomus cells or even if hypoxia induced glomus cell depolarization. Sharp electrode recording of resting potential of glomus cells often showed a relatively depolarized membrane potential, e.g. -10 mV (Acker and Pietruschka, 1984), -20 mV (Baron and Eyzaguirre, 1977) and -26 mV (Goldman and Eyzaguirre, 1984 Eyzaguirre et al., 1989). Moreover, the response to anoxia or cyanide was variable and of small magnitude with a depolarization in half the glomus cells and hyperpolarization in the other half of glomus cells (Eyzaguirre et al., 1989). Furthermore, in other studies, there was no correlation between the Nernst potential for K^+ and glomus cell membrane potential for clustered glomus cells (Zhang et al., 1995), suggesting that channels selective for K^+ were not a major factor in determining resting membrane potential. However, other observations suggested that K^+ channels or K^+ flux may be important since an increase in perfusate K^+ caused glomus cell secretion, presumably by cell depolarization and activation of voltage-dependent calcium currents (Obeso et al., 1992) and extracellular K^+ ion activity increased in the carotid body during hypoxia stimulation, suggesting a net flux from inside the cells (O'Regan and Acker, 1988). These complemented recent studies using tight-seal patch clamp recordings that identified K^+ currents present in glomus cells and identified several candidate K^+ currents which were altered by hypoxia.

Below, we will consider three different types of oxygen-sensitive K^+ currents and discuss their

possible roles in mediating hypoxia transduction in terms of the three responses: cell depolarization, glomus cell secretion and increase in nerve activity. In this discussion, there will be a tacit assumption that the mechanism of hypoxia transduction is the same across mammalian species, but this is unproved.

2. Types of oxygen sensitive K^+ currents

2.1. An A-like current

The first current, which was initially described in 1988 is the fast-inactivating, A-like current observed in rabbit glomus cells and which is inhibited by hypoxia (López-López et al., 1989, 1993). Since the current is activated by depolarization and it rapidly inactivates, fitting this current into the transduction schema requires a modification of the schema shown in Fig. 1. Instead of a relatively constant membrane potential, the glomus cell may have endogenous pacemaker-like activity, i.e. repetitive spiking activity. Inhibition of the A current is expected to reduce the post-spike hyperpolarization and enhance the endogenous spiking activity, thus leading to a net increase in calcium influx. Experimental evidence for the schema has been obtained in some cells (López-Barneo, 1996).

Critique: Several lines of observation argue against the central importance of such a current in hypoxia transduction: (1) The current does not appear to be present in glomus cells harvested from other species. In particular, it is not present in rat, cat or pig glomus cells (Chou and Shirahata, 1996; López-López et al., 1997). If it were central to hypoxia sensing by the carotid body then it would be anticipated that a similar mechanism would be shared across species; (2) Spontaneous spiking activity which is a central tenet of the theory has not been reported from studies using sharp electrode recordings (Eyzaguirre et al., 1989). Although these studies were generally designed to obtain intracellular recording, it would have been hard to miss spiking activity by hundreds or thousands of glomus cells when the electrode was positioned in the extracellular space; (3) Application of classical potassium channel blocking agents generally fail to emulate hypoxia. If the initial step in hypoxia transduction is an inhibition of a K^+ current then application of a channel blocking agent which targets the current would be expected to emulate hypoxia. The O_2 sensitive, A-current has been shown to be potently inhibited by low concentrations of 4-aminopyridine (4-AP) and tetraethylammonium (TEA) (López-López et al., 1993). However, for a number of years it has been known that these drugs cause little or any change in carotid body output. One example is from Moe et al. (1948) in which TEA was infused in anesthetized dogs to block the effect of exogenous acetylcholine (Fig. 2). (Their hypothesis dealt with acetylcholine and not K^+ channels.) TEA was effective in ablating the stimulating action of exogenous acetylcholine on ventilation, but had no effect on the respiratory stimulation by cyanide (Fig. 2). Recently, experiments targeted at K^+ channels were undertaken in intact carotid bodies or isolated glomus cells. Again, application of K^+ channel blocking agents

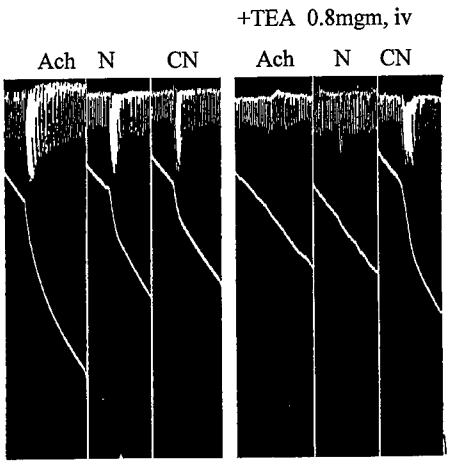

Fig. 2. TEA administration blocks the stimulatory action of nicotine injection but does not alter baseline respiration or response to cyanide. Respiration (top) and respiratory minute volume (spirometer) in an anesthetized dog before and during infusion of TEA. Note TEA infusion blocked the stimulatory action of exogenous acetylcholine on ventilation (panel 1, left; panel 1, right) but was without effect on effect on respiration or respiratory response to cyanide (panel 3, left; panel 3, right). (Reproduced with kind permission from *The Journal of Physiology*, Moe et al., 1948).

failed to excite the afferent nerve activity or block hypoxia excitation despite inhibition of glomus cell K^+ current (Figs. 3 and 4) (Donnelly, 1995). Similarly, in cat carotid body, in vivo, infusion of 4-AP caused little change in nerve activity or response to hypoxia (Pokorski and Lahiri, 1984). In isolated rat glomus cells, application of a cocktail of 10 mM TEA + 5 mM 4-AP failed to change resting membrane potential or increase intracellular calcium (Buckler, 1997). On a cautionary note, the oxygen-sensitive A current has only been described in rabbit cells while the observations with blocking agents were undertaken in rats, dogs and cats; (4) The dose/response of the hypoxia sensitivity appears to be inappropriate for mediating the chemoreceptor response. In two studies that focused on the dose/response of the A current to hypoxia, the current was fully inhibited at a P_{O_2} near to 90 Torr (Ganfornina and López-Barneo, 1991; López-López et al., 1993). In contrast, nerve activity from the intact organ increases when tissue P_{O_2} falls below 20 Torr (Rumsey et al., 1991), far below the response range of the A-current. The reason for the discrepancy is unclear, but may be partially related to the accuracy of the chamber P_{O_2} measurements in the cellular studies (Montoro et al., 1996).

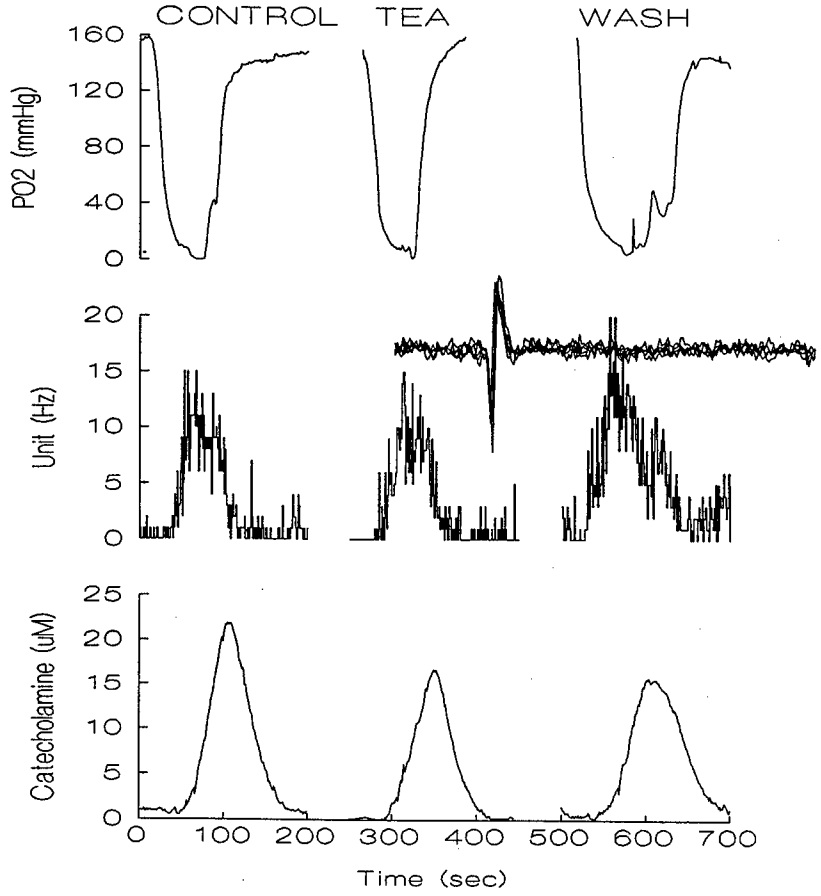

Fig. 3. Effect of K^+ channel blocker, TEA (20 mM) on nerve and catecholamine response to hypoxia. Top, superfusate P_{O_2}. Middle, Single fiber nerve activity. Insert: oscillographic tracing of nerve signal. Lower, free tissue catecholamine as assayed by carbon fiber voltammetry. Note: TEA did not stimulate secretion or nerve activity nor did it alter the action of hypoxia on catecholamine release.

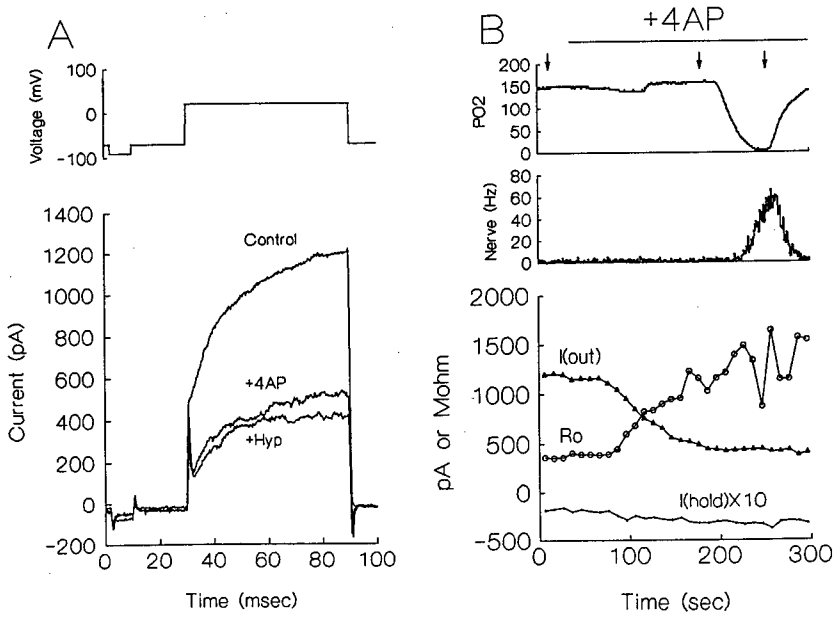

Fig. 4. Response of a glomus cell and nerve activity to 4-AP and hypoxia recorded from an intact rat carotid body: (A) Oscillographic current tracing during a step hyperpolarization from −60 to −80 mV and a step depolarization to +40 mV; (B) Time-dependent changes in nerve and cell characteristics. Note: Application of 4-AP decreased cell outward current but failed to change nerve activity or to block the response to hypoxia (Reproduced with kind permission from *The American Journal of Physiology*, Donnelly, 1995).

2.2. A BK-type, calcium dependent K^+ current

A second oxygen-sensitive K^+ current which was initially identified in rat glomus cells is a voltage-dependent, calcium dependent K^+ current, similar in characteristics to the BK-type (or large conductance) calcium-dependent K^+ current. This current is reversibly inhibited by hypoxia and by hypercapnia (Peers, 1990a,b,c; Peers and O'Donnell, 1990; Peers and Green, 1991; Wyatt and Peers, 1992; Wyatt et al., 1995). Since the activation voltage is ~ -20 mV, it is a unclear whether this current participates in determining resting membrane potential which is ~ -40 mV in these cells (Buckler and Vaughan-Jones, 1994; Wyatt et al., 1995). However, this appears to be the case since application of acute hypoxia (12–20 Torr) caused a depolarization of 8.6 mV under control conditions but no change in voltage following administration of charybdotoxin, a specific blocker of the BK-type Ik(Ca). Furthermore, under culture conditions of isolated rat glomus cells harvested from 20-day old rats, application of iberiotoxin, a selective blocker of this channel, enhanced catecholamine release from the cultured cells in the same way that hypoxia evoked catecholamine release (Jackson and Nurse, 1997). These responses may be specific for rat glomus cells since the BK-type current, although present in rabbit glomus cells, is not sensitive to hypoxia (Ganfornina and López-Barneo, 1992).

Critique: As for the argument dealing with the A-type current, the main criticism for the importance for this channel is that drugs which are expected to block this channel fail to emulate hypoxia. Thus, charybdotoxin fails to increase intracellular calcium in rat glomus cells (Buckler, 1997) and fails to increase baseline chemoreceptor afferent nerve activity in both rat carotid body (Cheng and Donnelly, 1995) and cat carotid body (Osanai et al., 1997), although there may be a potentiation of the nerve response during hypoxia stimulation (Pepper et al., 1995). Charybdotoxin is fairly specific agent in targeting the BK-type channel, but it is also expected that broader spectrum K^+ blocking agents such as TEA and 4-AP

would antagonize the oxygen sensitive K^+ current (Fig. 4). As referenced in the preceding section, in all cases in which this has been tested with 4-AP and TEA, the anticipated responses failed to be observed.

2.3. A 'leak', non-voltage dependent K^+ current

A third type of oxygen-sensitive K^+ current was recently observed in rat glomus cells (Buckler, 1997). This current contributes about half of the resting conductance of isolated rat glomus cells and is not voltage dependent. During hypoxia, the leak current is inhibited, resulting in a small depolarizing current ($\sim$7 pA in anoxia) to the cell. Unlike the other O_2 sensitive K^+ current, this leak current is not inhibited by classical K^+ channel blocking agents like TEA and 4-AP (Buckler, 1997). Of particular import, the dose/response of the current appears to be well correlated with the dose/response for changes in glomus cell calcium. Thus, the apparent binding affinity (Kd) for oxygen of the leak K^+ channel is 12 Torr (Buckler, 1997), a value close to the point at which intracellular calcium is found to rapidly increase ($\sim$8 Torr) (Buckler and Vaughan-Jones, 1994) and in the range of tissue P_{O_2} values for which nerve activity rapidly increases (Rumsey et al., 1991). In addition, metabolic blockers, which are known to be potent stimulating agents of peripheral chemoreceptor also appear to inhibit the same K^+ leak current (Buckler and Vaughan-Jones, 1998).

Critique: At present, the greatest limiting factor in establishing the importance of this leak channel is the lack of channel pharmacology, outside of the demonstration that it is not inhibited by the classical K^+ channel blocking agents, TEA, 4-AP and charybdotoxin. Thus, the important experiment of comparing pharmacologic inhibition of the channel with changes in nerve activity has not been performed. However, it may be important that barium causes a potent excitation of carotid chemoreceptor nerve activity (Gual and Stensaas, 1985; Donnelly, 1997), and barium is an inhibitor of some types of leak K^+ channels, for instance, the ORK1 channel (Goldstein et al., 1996).

A second factor or concern is the relatively small size of the depolarizing current caused by inhibition of the O_2-sensitive leak current. Anoxia caused $\sim$7 pA depolarization current (Buckler, 1997). For this current to have a major role in determining glomus cell membrane potential, the cell must have a fairly high input resistance. This is the case of several studies of isolated/dissociated glomus cells which have averaged around 4 GΩ input resistance (Duchen et al., 1988; Hempleman, 1996; Buckler, 1997). However, this has not always been the case since several patch-clamp studies have recorded values near 1 GΩ (Stea and Nurse, 1991; Donnelly, 1995). Even lower values were routinely recorded using sharp electrode recordings which measured input resistance of 174–253 MΩ in rat cells (He et al., 1991; Monti-Bloch et al., 1993), 64 MΩ in cat (Eyzaguirre et al., 1989) and 19–32 MΩ in rabbit (Acker and Pietruschka, 1984; Goldman and Eyzaguirre, 1984). The difference is probably not related to interactions with surrounding cells (perhaps through gap junction) since there was no difference in input resistance in isolated versus clustered cells when measured with sharp electrodes (Pang and Eyzaguirre, 1992). These lower input resistance values are, perhaps, not that unexpected. Values for specific membrane resistance for neurons is usually specified as 1000–5000 Ω/cm^2. For a spherical cell with a diameter of 12 μ, this yields an estimated input resistance of 221–1100 MΩ, very similar to measured values in some sharp-electrode and patch-clamp studies. If the same 'leak' depolarizing current was applied to a cell with an input resistance of 200 MΩ the estimated change in membrane potential would only be: 7 pA × 200 MΩ = 1.4 mV, and it would seem difficult to effect a transduction cascade with this level of stimulus encoding. (This should be considered as only a gross estimate of the depolarization magnitude. It assumes an identical membrane potential and electrochemical driving force for the O_2-sensitive leak current. A better estimate could be afforded by application of the GHK equation, but many of the permeability parameters as well as ion concentrations are not currently known.)

Further complicating the above picture are important observations by Stea and Nurse who observed a five-fold decrease in input resistance upon the addition of bicarbonate to the perfusate (average resistance in bicarbonate = 450 MΩ) (Stea and Nurse, 1991). This complemented previous work in their laboratory which identified a high conductance, anion-selective channel in glomus cells which can pass bicarbonate ion (Stea and Nurse, 1989). The conductance of a single anion channel was so large (~296 pS) that the opening of a single channel would be expected to cause a shunt resistance of 3 GΩ (1/296 pS), which would have reduced the anticipated depolarization by the 'leak' current by over 50%. Clearly, the evaluation of the purported depolarizing current is complicated and cannot be well resolved until the electrophysiologic state of the intact glomus cell is better understood.

3. Types of oxygen insensitive K^+ currents

In addition to K^+ selective currents which are modulated by hypoxia, glomus cells possess a number of oxygen insensitive K^+ currents. Rabbit cells have an small conductance calcium-dependent K^+ current which is activated by voltage and increased intracellular calcium above 100 nM (Duchen et al., 1988; Ganfornina and López-Barneo, 1992). While not directly sensitive to hypoxia, this current may be activated by the increased intracellular calcium which occurs during stimulation (Biscoe and Duchen, 1989). Rabbit cells, like rat cells, also have a large conductance K^+ current which is activated by depolarization and increased intracellular calcium (Ganfornina and López-Barneo, 1992). In inside–out excised membrane patches, channel activity was not dependent on oxygen level (Ganfornina and López-Barneo, 1992). Potassium currents have been recorded in rat and cat glomus cells, and in general, these currents are activated by depolarization and there is little time-dependent inactivation (Donnelly, 1993; Chou and Shirahata, 1996). Characterization of the oxygen-insensitive currents in terms of pharmacology and channel conductance has not been undertaken in these species.

4. Is depolarization an essential element of chemotransduction?

Central to the thesis that inhibition of an oxygen sensitive K^+ channel is a critical step in the hypoxia transduction cascade is that membrane potential changes in hypoxia. This appears to be clearly the case in some studies of isolated glomus cells using patch clamp recording (Buckler and Vaughan-Jones, 1994; Buckler, 1997). In rat cells recorded with the perforate patch technique, hypoxia caused a depolarization of 8.6 mV from a resting potential of −42.5 mV (Wyatt et al., 1995). However, depolarization is not a universal finding. In patch clamp studies of isolated rabbit cells, cyanide caused a membrane hyperpolarization, not depolarization (Biscoe and Duchen, 1989). Since cyanide is a potent stimulating agent of chemoreceptor nerve activity and causes an increase in glomus cell calcium, it would appear to have much in common with the transduction cascade for hypoxia, and this would suggest that depolarization is not a necessary step for the transduction cascade to occur. Complementing this observation is a recent report that depolarization is not an essential element for calcium influx through L-type calcium channels in hippocampal neurons during hypoxia (Nowicky and Duchen, 1998). If a similar mechanism is in place in glomus cells then depolarization would not be an essential step.

Further supporting this contention is the small or absent change in membrane potential during hypoxia recorded in some studies of glomus cells. Using sharp electrode impalement of isolated rat glomus cells, acute hypoxia caused a hyperpolarization in 64% of sample and a depolarization in only 29% of the sample (Pang and Eyzaguirre, 1992). Of these, the magnitude of the depolarization was relatively small (< 10 mV). As contrasted with isolated cells, cells in clusters in the intact or sliced organ generally (~60% of sample) depolarize with chemostimulation (Eyzaguirre et al., 1989; Pang and Eyzaguirre, 1992), but the magnitude is also small (< 10 mV in 90% of sample) (Pang and Eyzaguirre, 1992). Perhaps, even more important for our subject, depolarization was associated with a decrease in input resis-

tance, not the anticipated increase in resistance if the inhibition of a K^+ current accounted for the depolarization (Eyzaguirre et al., 1989).

5. Summary and future directions

Results of studies on isolated glomus cells have yielded several plausible steps for the initial step of hypoxia transduction, which appears to depend on calcium influx, and enhanced secretion. However, considerable uncertainty exists in the steps leading to calcium influx. Sharp electrode recordings often observed a relatively depolarized membrane potential and a highly variable change in membrane potential during hypoxic stimulation. In contrast, recent patch clamp recordings, generally of isolated cells, observed more hyperpolarized potentials and a more vigorous depolarization in response to hypoxia. Based on these patch clamp recordings, hypoxic transduction could be explained by several transduction schema in which transduction was initiated by inhibition of one of several K^+ currents: a transient current, a calcium-dependent current and a leak current.

Once these currents have been identified and characterized, the critical experiment becomes testing hypotheses based on the proposed transduction schema. One prediction is that application of pharmacologic blockers of the oxygen-sensitive channels should mimic hypoxic and lead to enhanced glomus cell secretion and enhanced nerve activity. In general, however, this has not been confirmed and blockers such as TEA, 4-AP and charybdotoxin are without major effect on organ function. Still, there are some notable exceptions, and enhanced glomus cell secretion was observed with high doses in 4-AP (Doyle and Donnelly, 1994) and application of iberotoxin, a blocker of the calcium dependent K^+ current (Jackson and Nurse, 1997). If K^+ currents which are sensitive to these blockers mediate the initial step in hypoxia transduction then we need to understand our failure to observe the expected excitation of afferent nerve activity. We would also need to better explain why nature solved the transduction problem differently in different species, i.e. the transient A-current has only been observed in rabbit glomus cells and why the BK current appears to be oxygen sensitive in the cat and rat but not in the rabbit.

Clearly there is much more to do. In particular, we need to better understand the electrophysiologic state and function of the intact glomus cells. Measurements of the input resistance of glomus cells has spanned the range from ~ 19 to 4000 $M\Omega$, and identification of currents which may mediate cell depolarization will depend on understanding both the magnitude of the current and the background conductance against which it is working. At present we have little information in this regard.

In conclusion, a role in hypoxia transduction for an oxygen sensitive K^+ channel is not firmly established. However, progress appears be moving forward rapidly with good synergy between the identification of candidate K^+ currents and experiments in other laboratories to see if the anticipated changes in cells secretion or afferent nerve activity takes place. This promises to resolve the issue in the near future.

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RESPIRATION PHYSIOLOGY

NO and CO as second messengers in oxygen sensing in the carotid body

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Abstract

It is being increasingly appreciated that nitric oxide (NO) and carbon monoxide (CO) are synthesized in mammalian cells and that they function as second messengers. The purpose of this article is to highlight the current information on NO and CO in the carotid body and discuss their significance in oxygen chemoreception. The NO synthesizing enzyme, nitric oxide synthase, is localized to nerve fibers and vascular endothelium in the carotid body. In vitro biochemical assays have shown that acute hypoxia inhibits NO synthase activity in carotid body extracts. Prolonged hypoxia up-regulates mRNA's encoding neuronal and endothelial NO synthases in the carotid body. Physiological studies have shown that NO is inhibitory to the carotid body sensory activity and mediates efferent inhibition. The actions of NO are in part mediated by its effects on glomus cells, wherein NO modulates Ca^{2+} channel activity and affects $[Ca^{2+}]_i$. The carotid body also uses another highly related gas as a second messenger, carbon monoxide (CO). The enzyme responsible for CO biosynthesis, heme oxygenase-2, is localized to glomus cells. CO, like NO, also exerts an inhibitory influence on sensory activity. Some of the actions of CO are mediated by altering Ca^{2+} channel activity and $[Ca^{2+}]_i$ in glomus cells. Molecular oxygen is essential for biosynthesis of NO and CO. Under normoxia, basal levels of NO and CO act as amplifiers of molecular oxygen and keep the sensory discharge low. During hypoxia, decreased synthesis of NO and CO may contribute in part to the augmentation of sensory discharge.

Keywords: Carbon monoxide, glomus cell; Control of breathing, hypoxia, carotid body; Hypoxia, carotid body, signal transduction; Ion channels, Ca^{2+}, carotid body, NO; Nitric oxide, glomus cell

1. Introduction

Several lines of evidence suggest that neurotransmitters play an essential role in the transmission of the hypoxic stimulus to nerve encoded impulses in the carotid sinus nerve (Fidone and

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González, 1986; Prabhakar, 1994). The carotid body contains several classes of neurotransmitters, including biogenic amines (e.g. dopamine, norepinephrine, acetylcholine) and neuropeptides (e.g. enkephalins, substance P, atrial natriuretic peptide). Recent studies suggest that carotid bodies also synthesize gas molecules such as nitric oxide (NO) and carbon monoxide (CO), which may function as chemical messengers (Prabhakar, 1994). However, unlike biogenic amines and peptides, NO and CO are not stored in vesicles. Once generated, they rapidly diffuse to neighboring cells and are inactivated with a half-life of only a few seconds. More importantly, NO and CO share some common properties with molecular oxygen. For instance, like oxygen, both NO and CO are gas molecules and bind to heme, perhaps with greater affinity than oxygen (Abu-soud et al., 1996; Migita et al., 1998). Many of their biological actions are coupled to activation of heme containing proteins and are regulated by the redox status of the cell (Moncada et al., 1991; Snyder, 1992). These similarities with molecular oxygen prompted our interest in examining their function in the carotid body. The purpose of this article is to highlight some of the recent studies on NO and CO in the carotid body and briefly discuss their significance in oxygen chemoreception.

2. Nitric oxide (NO)

2.1. Distribution of NO synthases and evidence for generation of NO in the carotid body

In mammalian cells, NO is generated during the enzymatic conversion of L-arginine to L-citrulline and this reaction is catalyzed by the enzyme NO synthase (NOS). Three isoforms of NOS have been isolated: neuronal, endothelial and inducible (Moncada et al., 1991). In the carotid body the neuronal isoform of NOS is found in the nerve plexuses innervating the chemoreceptor tissue (Prabhakar et al., 1993; Wang et al., 1993; Fig. 1). The following lines of evidence suggest that NOS containing nerve fibers are of sinus nerve origin. First, NOS positive fibers are present in the sinus nerve and chronic ablation of sinus nerve abolishes NOS-positive fibers in the carotid body.

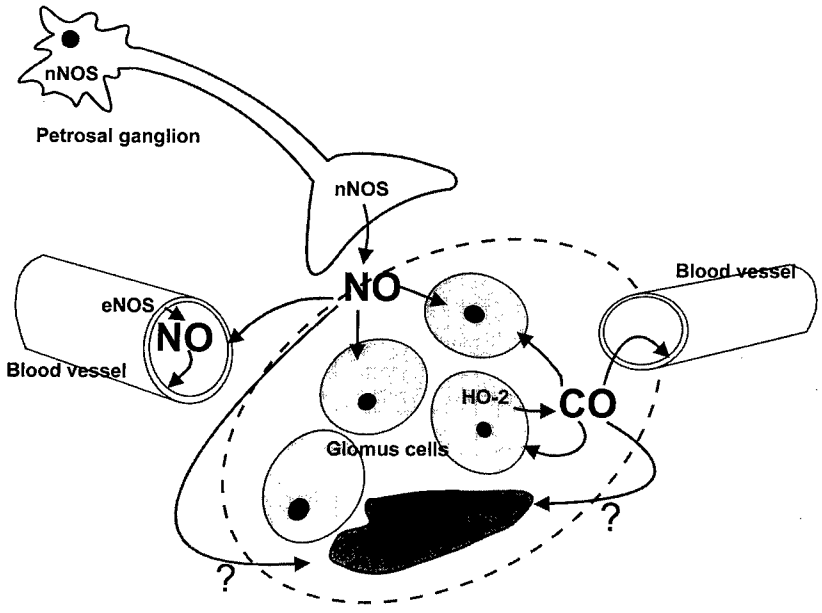

Fig. 1. Schematic presentation of localization and possible sites of action of nitric oxide (NO) and carbon monoxide (CO) in the carotid body. nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; HO-2, heme oxygenase 2.

Secondly, NOS containing neurons are present in the petrosal ganglion, which provides sensory innervation to the carotid body (Wang et al., 1993; Fig. 1). In addition, some ganglion cells in the carotid body also express neuronal NOS (Wang et al., 1993; Grimes et al., 1995). The endothelial isoform of NOS is expressed in blood vessels of the carotid body (Wang et al., 1993). Whether or not glomus cells express NOS remains uncertain. There is no evidence for induction of inducible isoform of NOS in the carotid body.

2.2. Biochemical studies of NOS in the carotid body and evidence for inhibition of NOS activity by acute hypoxia

The enzyme activity of NO synthase was measured by [^{3}H] citrulline assay in the cat (Prabhakar et al., 1993; Wang et al., 1994) and rat carotid bodies (Wang et al., 1993). Omission of Ca^{2+} from the reaction medium inhibited enzyme activity suggesting that NOS in the carotid body is primarily of the constitutive type. Furthermore, addition of NOS inhibitors to the reaction medium prevented conversion of arginine to citrulline, further supporting the presence of NOS (Wang et al., 1994). NOS is a heme-containing enzyme and requires molecular oxygen for its activation. In vitro studies have shown that hypoxia inhibits NOS activity (Rengasamy and Johns, 1991). The apparent Km values for oxygen were 23 ± 2.8 and 7.7 ± 1.6 μM for neuronal and endothelial NOS, respectively (Rengasamy and Johns, 1996). We have measured NOS activity in carotid body extracts under three levels of oxygen in the reaction medium (Prabhakar et al., 1993). Basal NOS activity under normoxia (P_{O_2} of the medium ~ 152 mmHg) averaged 1.94 ± 0.14 pmol/min/mg of protein. Lowering the P_{O_2} in the reaction medium to 54 and 32 mmHg reduced NOS activity to 1.21 ± 0.12 and 0.57 ± 0.17 pmol/min/mg of protein, respectively. These observations are consistent with the idea that hypoxia inhibits NOS activity in the carotid body. Wang et al. (1994), on the other hand, concluded that hypoxia has no effect on NOS activity in the carotid body. These investigators harvested carotid bodies from animals that were exposed to hypoxia prior to measuring NOS activity under normoxic conditions. The effects of hypoxia on NOS are quickly reversible. Therefore, although the tissues were removed from hypoxic animals, it is not surprising that NOS activity was the same as controls since enzyme activity was assayed in medium enriched with oxygen. Thus, the seemingly contradictory results by Wang et al. (1994) are most likely due to the experimental conditions under which NOS activity was measured. However, further studies with direct measurements of NO in the intact carotid bodies are necessary to establish whether hypoxia decreases NO levels in the intact organ.

2.3. Hypoxia up-regulates NOS gene expression in the carotid body

Recent studies suggest that oxygen can regulate NOS gene expression. We reported previously that hypobaric hypoxia up-regulates neuronal NOS mRNA in central and peripheral neurons (Prabhakar et al., 1996). Similarly, Shaul et al. (1995) observed up-regulation of neuronal and endothelial NOS mRNA's in rat lungs following 1–3 weeks of hypoxia. The fact that oxygen can regulate NOS gene expression prompted us to examine the effects of hypobaric hypoxia on neuronal and endothelial NOS mRNAs in the carotid bodies (Prabhakar et al., 1999). Adult rats of either sex were exposed to 0.4 ATM for 12 h or 15 days. Following the hypoxic exposure, carotid bodies were harvested and mRNA's encoding neuronal and endothelial NOS were analyzed by RT-PCR assay along with β-actin, which served as control. Neuronal and endothelial NOS mRNA's could readily be detected under basal normoxic conditions. Exposure to 12 h of hypoxia increased endothelial NOS mRNA three-fold, whereas neuronal NOS was unaltered. Furthermore, 15 days of hypoxia resulted in a nine-fold elevation in neuronal NOS mRNA with no changes in endothelial NOS mRNA (Prabhakar et al., 1999). These observations suggest that hypoxia stimulates NOS gene expression in the carotid bodies. Kusakabe et al. (1998) examined the effects of long term hypoxia (10% O_2 for 3 months) on NOS immunoreactivity in rat carotid

bodies. They found decreases in NOS immunoreactive nerve fibers in the carotid body following 3 months of hypoxia. It may be that the effects of hypoxia on NOS expression in the carotid bodies may depend on the duration. Thus, relatively shorter durations of hypoxia (e.g. hours to weeks) stimulate, whereas longer durations of hypoxia lasting several months down regulate NOS in the carotid body. Perhaps, the most important question to address is how the changes in NOS expression affect carotid body function during hypoxia.

2.4. NO is an inhibitory chemical messenger in the carotid body

2.4.1. Pharmacological studies

Various analogues of arginine function as inhibitors of NOS and offer valuable tools to investigate the significance of NO in various physiological systems (Moncada et al., 1991). We previously reported that L-nitro-ω-arginine (L-NNA), a potent inhibitor of NOS, augments the sensory discharge of the cat carotid bodies in vitro (Prabhakar et al., 1993). The effects of L-NNA were concentration-dependent and reversed by L- but not D-arginine. These findings were confirmed by independent studies by other investigators (Chugh et al., 1994; Wang et al., 1994). These observations suggested that NO is continuously produced under basal conditions and it exerts an inhibitory influence on sensory discharge. The notion that NO is inhibitory to carotid body activity is further supported by the finding that nitrosyl compounds that liberate NO (NO donors) reduced carotid body activity (Chugh et al., 1994; Wang et al., 1994). NOS inhibitors also augmented the carotid body response to hypoxia in isolated carotid bodies (Wang et al., 1994) as well as in anesthetized animals (Trezbski et al., 1995). Taken together, these observations suggest that endogenously generated NO acts as an inhibitory chemical messenger in the carotid body.

2.4.2. Involvement of NO in efferent inhibition in carotid body

Biscoe and Sampson (1968) recorded spontaneous centrifugal neural activity from the central stump of the carotid sinus nerve, suggesting an efferent pathway in the carotid sinus nerve. Subsequent studies by Neil and O'Regan (1971), Fidone and Sato (1970) demonstrated that electrical stimulation of the peripheral cut end of the carotid sinus nerve inhibited spontaneous chemoreceptor activity recorded from nerve filaments from an otherwise intact sinus nerve. This phenomenon is referred to as 'efferent inhibition'. However, mechanism(s) underlying efferent inhibition have not been investigated. Localization of NOS in the nerve fibers belonging to the sinus nerve as well as the fact that NO is inhibitory to the sensory discharge, led us to suggest that NO mediates efferent inhibition (Prabhakar et al., 1993). Wang et al. (1995) examined the effects of NOS inhibitor on efferent inhibition in the isolated cat carotid body preparation. They found that NOS inhibitor prevented efferent inhibition. These observations suggest that NO mediates efferent inhibition in the carotid body.

2.4.3. Studies on mice with targeted disruption of NO synthase isoforms: evidence for the actions of NO in the carotid body depend on the source of its production

The biological actions of NO seem to depend on the source of its production. For example, during focal ischemia, NO generated by the neuronal isoform of NOS exerts a toxic effect on neurons; whereas NO produced by endothelial NOS confers toxic resistance to neurons (Huang et al., 1994). Whether actions of NO on the carotid body also depend on the source of its production is not known. As described above, much of the information on the role of endogenous NO comes from the studies with NOS inhibitors, which can not distinguish between neuronal and endothelial NOS. Huang et al. (1993) developed transgenic mice with targeted disruption of neuronal and endothelial NOS. These mutant mice offer an excellent animal model for distinguishing the roles of NO generated by neuronal and endothelial NOS. Kline et al. (1998) examined peripheral chemoreceptor sensitivity in mutant mice deficient in neuronal NOS. They found that the respiratory response to brief hyperoxia (Dejour's test) and to cyanide (a potent chemoreceptor stimulant) were more pronounced

in mutant mice, suggesting augmented peripheral chemoreceptor sensitivity. Interestingly, mice deficient in endothelial NOS exhibited reduced peripheral chemoreceptor sensitivity and a blunted ventilatory response to hypoxia (Kline et al., 1999). Endothelial NOS knock-out mice had higher blood pressure compared to wild type controls. It is possible that chronic vasoconstriction, as evidenced by high blood pressure, might have rendered the carotid bodies insensitive to hypoxia. In this context it is worth noting that there are studies indicating blunted peripheral chemoreceptor sensitivity in hypertensive humans (Tafil-Kalwe et al., 1989) and experimental animals (Przybylski et al., 1982). None-the-less, these studies with mutant mice with disrupted NOS function support the idea that NO generated by NOS is an important physiological regulator of carotid body activity and suggest that NO actions depend on the source of its production.

2.5. Mechanism(s) and site(s) of action of NO in the carotid body

2.5.1. Evidence for cGMP-dependent and independent mechanisms

NO, once released, can affect carotid body sensory activity by a direct action on the glomus cells acting as a retrograde messenger as it does elsewhere in the nervous system (Snyder, 1992). In addition, NO may affect the sensory discharge by changing carotid body blood flow via its action on vascular smooth muscle cells (Fig. 1). Furthermore, there are a number of pathways through which NO can exert its actions in the carotid body (Fig. 2). One such pathway is via activation of the heme containing guanylate cyclase and subsequent elevation in cellular levels of cyclic guanosine monophosphate (cGMP; Snyder, 1992). Consistent with such a notion is the finding that NOS inhibitor markedly reduces (Prabhakar et al., 1993), whereas NO donors increase cGMP levels in the carotid body (Wang et al., 1995). The relative contribution of the cGMP pathway to inhibition of sensory discharge by NO, however, remains to be investigated. NO signaling can also encompass the actions of other naturally occurring NO derivatives, such as *S*-nitrosothiols,

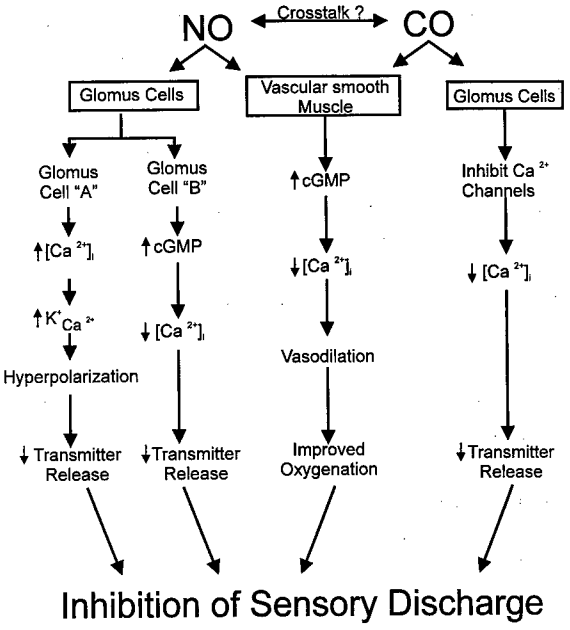

Fig. 2. Schematic illustration of possible mechanisms of action of NO and CO in the carotid body.

whose actions depend on the redox environment of the cell (Stamler, 1994; Campbell et al., 1996).

Ca^{2+} channels play an important role in carotid body chemoreception, and Ca^{2+}-dependent neurotransmitter release from glomus cells is believed to be an obligatory step in the transduction of the hypoxic stimulus (González et al., 1992). Hatton and Peers (1996) found no effect of the NO donor SNAP, on Ca^{2+} currents in glomus cells from rat carotid bodies. In contrast, Summers et al. (1999) observed inhibition of Ca^{2+} currents by NO donors in rabbit glomus cells. They reported that the NO donors, sodium nitroprusside (SNP) and spermine nitric oxide (SNO), inhibited Ca^{2+} currents in a voltage-independent manner. Carboxy-PTIO, a NO scavenger, prevented the inhibition of Ca^{2+} currents by NO donor. In contrast, NO did not affect the outward K^+ current. Furthermore, inhibition of the Ca^{2+} current by NO appears to be a direct effect on the Ca^{2+} channel protein, because it could be abolished by *N*-ethylmalemide, which prevents nitrosylation of proteins by NO. Finally, Summers et al. (1999) also observed that the effects of NO are

confined primarily to L-type Ca^{2+} channels. The lack of NO effects on rat glomus as reported by Hatton and Peers (1996) may conceivably be due to a species difference and/or to differences in the experimental conditions. None-the-less, these studies suggest that one mechanism by which NO exerts its actions at the carotid body may be a direct inhibition of Ca^{2+} current.

The finding that NO modulates Ca^{2+} currents in glomus cells prompted us to examine whether NO affects intracellular calcium $[Ca^{2+}]_i$ in rabbit glomus cells. Of the 114 cells tested, the NO donor SIN-1 decreased $[Ca^{2+}]_i$ in 23 cells (20%), whereas it increased in 57 cells (50%). The remaining 20 cells showed no change. The NO scavenger carboxy PTIO prevented NO-induced changes in $[Ca^{2+}]_i$. Superoxide dismutase (SOD) had no effect, suggesting that the actions of NO donor are due to generation of NO rather than superoxide ions. Removal of extracellular Ca^{2+} did not prevent NO-induced increases in $[Ca^{2+}]_i$ indicating that NO mobilizes Ca^{2+} from internal stores. This increase in $[Ca^{2+}]_i$, may activate Ca^{2+}-dependent K^+ channels, which in turn may contribute to NO-induced inhibition of the sensory discharge (Fig. 2). While these possibilities remain to be further studied, these results suggest that NO has complex effects on $[Ca^{2+}]_i$ in glomus cells.

3. Carbon monoxide (CO)

3.1. Distribution of heme oxygenase II in the carotid body

It was once thought that CO is a biologically indifferent gas like hydrogen. Now, it is fairly well established that CO is synthesized in mammalian cells during degradation of heme by the enzyme heme oxygenase (HO) and the reaction requires molecular oxygen. NADPH and cytochrome P-450 reductase are essential co-factors (Maines, 1997). Two distinct isoforms of heme oxygenases have been characterized, an inducible HO-1 and a constitutively expressed HO-2. Of the two isoforms, HO-2 is the one that is predominantly expressed in neuronal cells (see Prabhakar, 1998 for reference). The distribution of HO-2 has been examined in carotid bodies of cats and rats (Prabhakar et al., 1995). HO-2 like immunoreactivity was found in many glomus cells, however, no immunoreactivity was evident either in the nerve fibers or in the supporting cells (Fig. 1). On the other hand, HO-1 like immunoreactivity was not evident in the carotid body. These observations indicate that glomus cells of the carotid body express HO-2.

3.2. Evidence for CO as an inhibitory chemical messenger in the carotid body

To assess the functional significance of HO-2 in the carotid body, we examined the effects of Zn-protoporphyrin-9 (ZnPP-9), a potent inhibitor of HO, on carotid body sensory discharge in vitro (Prabhakar et al., 1995). As little as 0.3 μM ZnPP-9 augmented the sensory discharge, with maximal excitation at 3 μM. On the other hand, comparable concentrations of CuPP-9, a protoporphyrin that has negligible effects on HO did not excite carotid body activity. Exogenous administration of CO reversed the augmentation of sensory discharge by ZnPP-9. These observations suggest that endogenously generated CO, like NO and oxygen, also inhibit carotid body sensory activity.

Yang et al. (1998) examined the effects of SnPP-9, another potent inhibitor of HO, on respiratory responses to hypoxia and hypercapnia in anesthetized rats. These investigators found that SnPP-9 enhanced respiratory responses to hypoxia but not to CO_2. Bilateral sectioning of carotid sinus nerves abolished the effects of SnPP-9, suggesting that the effects of HO inhibitor are mediated by its action on carotid bodies. Recently, we also examined the ventilatory response to hypoxia in mice deficient in HO-2. Respiratory augmentation in response to hypoxia (12% O_2) was more pronounced in HO-2 knockout mice compared to wild type controls, and bilateral sectioning of carotid sinus nerves abolished the effects of hypoxia (Kline et al., 1999, unpublished data). These observations are consistent with the view that endogenously generated CO from HO-2 is a physiological regulator of carotid body activity.

3.3. Inhibitors of heme oxygenase increase $[Ca^{2+}]_i$ in glomus cells: evidence for activation of Ca^{2+} channels

The localization of HO-2 to glomus cells suggests that CO may act back on the same cell in an autocrine manner, or may influence nearby cells in a paracrine manner. Overholt et al. (1996) examined the effects of HO inhibitors on $[Ca^{2+}]_i$ in glomus cells. In response to ZnPP-9, $[Ca^{2+}]_i$ increased in glomus cells. This increase in $[Ca^{2+}]_i$ could be prevented by exogenous administration of CO, as well as by removal of extracellular Ca^{2+}. ZnPP-9 also augmented Ca^{2+} currents in a few glomus cells tested. These observations indicate that endogenous CO affects $[Ca^{2+}]_i$ and thus may regulate transmitter release (Fig. 2). In addition, CO could also influence carotid body activity by acting as a vasodilator, thereby enhancing the oxygenation of the glomus tissue (Fig. 2).

4. Significance of NO and CO in oxygen chemoreception in the carotid body

The observations described thus far indicate that carotid body activity is under a tonic inhibitory influence from NO generated by the nerve endings and blood vessels, and CO produced by glomus cells. How might NO and CO contribute to O_2 chemoreception in the carotid body? First, synthesis of both of these gas molecules requires molecular oxygen (Moncada et al., 1991; Maines, 1997). In addition, biochemical studies indicate that NOS activity is sensitive to hypoxia over a physiologically relevant range (Rengasamy and Johns, 1991, 1996; Abu-soud et al., 1996). Likewise, the affinity of the heme-hemeoxygenase complex for oxygen is 30–90 fold greater than myoglobin (Migita et al., 1998). It has been proposed that certain regions of the carotid body are normally perfused with plasma (plasma skimming), and chemoreceptor cells are exposed only to dissolved oxygen (Acker, 1980). Since NOS and HO operate over a wide range of oxygen, it is possible that endogenously generated NO and CO amplify the effects of dissolved oxygen, and being inhibitory to the sensory discharge, keep the carotid body activity low under normoxic condition. Since low oxygen reduces the enzyme activities of NOS and HO, the increased sensory discharge during hypoxia could in part be due to decreased production of NO and/or CO, thus releasing this inhibition. While this hypothesis is attractive, further experiments are necessary to establish whether or not hypoxia decreases NO and CO in the intact carotid bodies. CO may potentially regulate the actions of NO in the nervous system (Prabhakar, 1998). Is there any interaction between NO and CO in the carotid body, and if so what impact the interactions have on carotid body function remains to be explored.

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Redox-dependent binding of CO to heme protein controls P_{O_2}-sensitive chemoreceptor discharge of the rat carotid body

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Abstract

Simultaneous recordings of chemoreceptor discharge and redox state of cytochromes have been carried out on the rat carotid body in vitro under the influence of carbon monoxide (CO) in order to identify the primary oxygen sensor protein controlling transmitter release and electrical activity. CO excites in a photolabile manner chemoreceptor discharge under normoxic conditions and inhibits under hypoxic conditions probably by binding to heme proteins. We hypothesize that type I cells and adjacent nerve endings of the carotid body tissue have a different apparatus with oxygen sensing heme proteins to cooperate for the generation of peripheral chemoreceptor response. Transmitter release from type I cells might be established in a redox dependent manner whereas membrane potential of nerve endings might be controlled by a heme coupled to ion channels.

Keywords: Redox-dependent binding; Chemoreceptor discharge; Rat carotid body; Ion channels

1. Introduction

The oxygen sensing signal cascade enabling cells to regulate electrical activity and gene expression dependent on enviromental P_{O_2} is supposed to consist of a heme protein which senses oxygen and transfers this signal to ion channels or transcription factors (long-term effects) either as a kinase, by reactive oxygen species (ROS) generation or by allosteric conformation changes (for review see Acker, 1994). The generation of action potentials dependent on oxygen in the carotid body is supposed to involve P_{O_2}-sensitive potassium channels which lead under hypoxia to membrane depolarisation of type I cells and a rise of the intracellular calcium level with a subsequent transmitter release to excite synaptically con-

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nected nerve endings (López-Barneo et al., 1993; Peers, 1997). However recent work by Donnelly (1997), Lahiri et al. (1998) and Roy et al. (1998) questioned this model since blocking agents of the P_{O_2} sensitive potassium channels as found in studies on isolated type I cells failed to mimic hypoxia neither stimulating chemoreceptor nerve activity nor enhancing catecholamine secretion of the rat carotid body. The importance of the closure of these potassium channels under hypoxia was not consistent with the observations as published by Delpiano and Acker (1989) showing a significant increase of extracellular potassium in the carotid body during hypoxia. The gap between studies on type I cells and nervous discharge was furthermore substantiated by studies of Lahiri et al. (1998) showing that low Ca^{2+} (0.1 mM) and high Mg^{2+} (6 mM) which decreased glomus cell K^+ conductance (Hatton et al., 1997) but inhibited the neuromuscular transmission (Katz, 1969), did not cause chemoreceptor excitation nor did it block hypoxic neurotransmission (Lahiri et al., 1998)

In order to clarify the role of single carotid body cell elements during hypoxia and the nature of the oxygen sensing heme protein we continued with experiments as described by Buerk et al. (1997) and Wilson et al. (1994) using carbon monoxide (CO) to excite chemoreceptor discharge of the rat carotid body under different redox conditions as controlled by light absorption photometry of mitochondrial and non mitochondrial cytochromes (Lahiri, 1994; Acker and Xue, 1995). We will show that CO and oxygen compete for binding to a heme protein which is hypothesized to be located in the cell membrane of the sinus nerve endings and controls the pore size of ion channels and thereby action potential generation presumably by conformation changes.

We conclude

1. that beside mitochondrial cytochromes (Mills and Jöbsis, 1972; Duchen and Biscoe, 1992; Wilson et al., 1994) and non mitochondrial cytochrome b_{558} (Cross et al., 1990; Kummer and Acker 1995; Youngson et al., 1997) a third heme protein is involved in oxygen sensing
2. and that beside studies on type I cells, nerve endings have to be investigated for their oxygen sensing properties.

2. Materials and methods

2.1. Experimental protocol

Rats with a body weight between 150 and 200g were anesthetized with 0.45 ml/100g body weight of a 25% urethran and 4% chloralose solution. The carotid artery bifurcations were exposed and the sinus nerve cut near its junction with the glossopharyngeal nerve. The carotid artery bifurcation was dissected and placed in chilled, oxygenated isotonic salt solution (containing in mM: NaCl 128, KCl 5,6, glucose 27,5, Hepes 17, $CaCl_2$ 2,1). For clearing red cells of the carotid body carotid arteries were perfused with oxygenated isotonic salt solution for ten minutes after heparinising the animal by injecting liquemin (1.3 USPE/g) into the jugular vein. After dissection of the bifurcation oxygenated isotonic salt solution was injected into the occiptal artery to clean furthermore the carotid body vasculature from red cells. Carotid body and sinus nerve were carefully liberated from connective tissue and cut out from the bifurcation.

2.2. Light absorption photometry

Isolated carotid bodies with corresponding sinus nerve were located in a superfusion chamber on a small opaque bench with little holes of similar diameter as the carotid body. Superfusion was performed as described by Delpiano and Acker (1985). Briefly: isotonic salt solution (containing in mM: NaCl 128, KCl 5,6, glucose 27,5, Hepes 7, $NaHCO_3$ 10, $CaCl_2$ 2,1) was equilibrated with different O_2/CO_2 mixtures in order to adjust oxygen tension to various levels at a pH of about 7.40. The flow rate through the chamber was 60 ml per min. Carotid bodies were supplied symmetrically with nutrients by this procedure. The temperature was maintained at 36°C. The superfusion chamber was mounted on the stage of a light microscope (Olympus, Hamburg, FRG) for light absorption measurements as described by Ehleben et al. (1997) and Porwol et al. (1998). White light from a halogen bulb (12 V, 100 W) transilluminating the carotid body for 10 s each minute by opening a shutter passed to an objec-

tive (40 ×) and was analysed for absorption changes at different wavelengths by a photodiodearray spectrophotometer (MCS 210, Zeiss, Köln, FRG) connected to the third ocular of the microscope trinocular head via a light guide. Difference spectra were obtained by firstly recording in superfusion medium equilibrated with 28.8% O_2, 4% CO_2 and 67.2% N_2 (aerobic steady state) which was automatically substracted from the spectra under reducing conditions by equilibrating with 4% CO_2 and different concentrations of N_2, O_2 and CO. The adjustment of the gas concentrations was performed with two gas mixing pumps (Wösthoff, Bochum, FRG). The recorded difference spectra were evaluated, deconvoluted and visualized using the software package TechPlot (Dr.Dittrich, Braunschweig, FRG).

2.3. Sinus nerve activity

Chemoreceptor afferent recordings were obtained as previously described by Donnelly and Kholwadwala (1992). Briefly: a glass microelectrode (tip diameter 130–160 μm) filled with isotonic salt solution was used to suck in the cut end of the sinus nerve. The electrode was connected to a differential preamplifier (Model DAM5A, WPI-Instruments, Berlin, FRG) which was coupled to a window discriminator (Model 121, WPIInstruments) with a subsequent home made rate meter. Counting rate of the action potentials as well as transillumination time of the carotid body were stored with the aid of a home made computer program. The action potentials could be visualized on an oscilloscope (TDS 210, Tektronix, Köln, FRG).

2.4. Statistical analysis

Data are presented as mean ± S.D. For statistical analyses the Student's *t*-test was performed. Differences were considered significant when the pvalue was ± 0.05.

3. Results and discussion

Light absorption photometry is an appropriate method to study the redox state of the superfused carotid body tissue in vitro under various experimental conditions. In case of a carotid body containing red cells simultaneous recorded chemoreceptor discharge can easily be related to the changed oxygen supply conditions during superfusion with N_2 equilibrated medium as measured by the oxygenation state of hemoglobin described in Fig. 1a. On the left side chemoreceptor discharge is shown to increase and decrease in close relationship to hemoglobin spectra drawn on the right side getting deoxygenated and reoxygenated at time points which can easily be related to chemoreceptor discharge with the aid of the little bars indicating opening of the shutter. The bar marked as N_2 indicates the time when equilibration of the superfusion medium is switched from 28,8% O_2, 4% CO_2, 67,2% N_2 to 96% N_2, 4% CO_2. The first small increase of sinus nerve activity coincides with a visible hemoglobin deoxygenation which reaches the highest degree when chemoreceptor discharge levels off after a transient peak. Reoxygenation is followed by a fast chemoreceptor decline reaching control when hemoglobin is still somewhat deoxygenated. This record reveals the complex interaction between enviromental factors like oxygen concentration of carotid body tissue and generation of action potentials. It seems to be very obvious from the non linear interaction that nervous discharge is not only determined by oxygen but other factors like extracellular potassium (Delpiano and Acker, 1989), pH (Delpiano and Acker, 1985) and transmitter release characteristics (for review see Gonzalez et al., 1994) might contribute in an unknown manner to the final response curve. The main goal of present work however is the identification of the oxygen sensing heme protein interacting with oxygen to generate action potentials. The mitochondrial cytochrome aa_3 partly with an unusual low P_{O_2} affinity has been described as oxygen sensing protein in carotid body type I cells (Mills and Jöbsis, 1972; Duchen and Biscoe, 1992; Wilson et al., 1994), heart (Budinger et al., 1998) and liver (Chandel et al., 1997). In these, respiratory chain has been directly connected with chemosensory discharge (Mulligan et al., 1981; Wilson et al., 1994). A low out put form of cytochrome b_{558} probably an isoenzyme of the

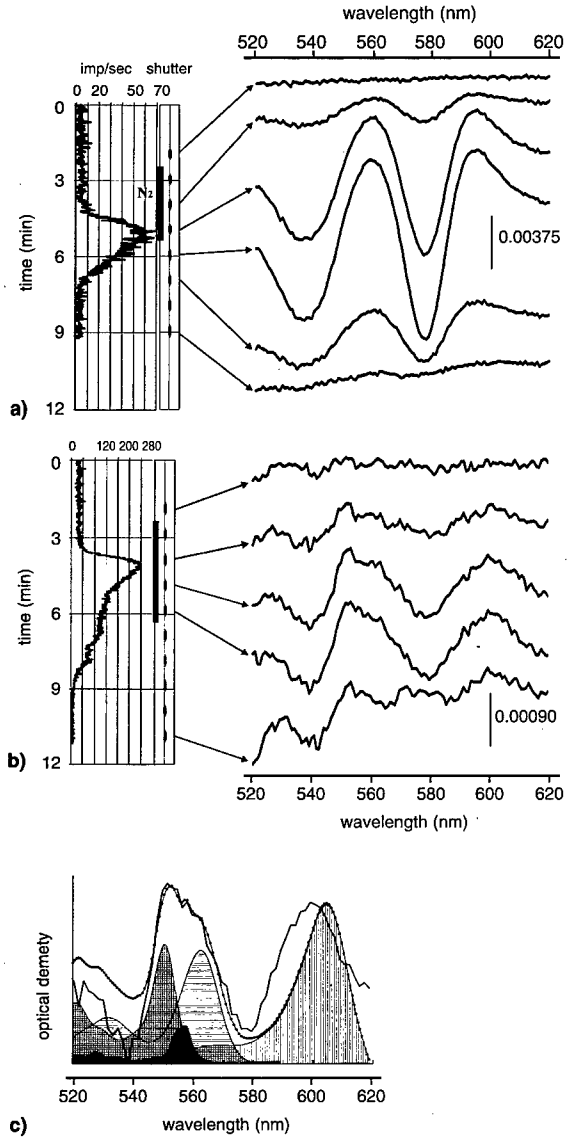

Fig. 1. Simultaneous recordings of chemoreceptor discharge (imp/s) as shown on the left side and light absorption spectra shown on the right side in a wavelength range between 520 and 620 nm. Hypoxia indicated by a bar marked as N_2 leads to deoxygenation of hemoglobin (a) or to reduction of cytochromes (b) of carotid body tissue accompanied by chemoreceptor discharge excitation. The transillumination time to record light absorption spectra is shown by little bars marked as shutter. The most reduced light absorption spectrum of the cytochromes is deconvoluted (c) using redox spectra of isolated cytochrome c peaking at 550 nm (+ +), cytochrome b_{558} (*), cytchrome b_{563} (=) and cytochrome aa_3 peaking at 605 nm (||). Light absorption changes of the specta of the isolated cytochromes are relative values that were calculated to fit the experimental curve (solid line) as close as possible by a superposition curve (open circles).

high put neutrophil NADPHoxidase is reported to be the primary oxygen sensing cytochrome in carotid body type I cells (Cross et al., 1990; Kummer and Acker, 1995; Youngson et al., 1997), neuroepithelial body cells (Wang et al., 1996), smooth muscle cells of the pulmonary vasculature (Marshall et al., 1996) and hepatoma cells (Ehleben et al., 1997). However, H_2O_2 generated by NADPH-oxidase which has an effect on K^+ channel but not consistent with P_{O_2} effects. For example, exogenously applied H_2O_2 which increased some K^+-current in xenopus oocytes had no responses to changes in P_{O_2} (Vega-Saenz de Meira and Rudy, 1992). Osanai et al. (1997) did not find evidence for H_2O_2 role in carotid chemoreceptor activity in contrast to Acker et al. (1992) describing a degradation of H_2O_2 by glutathion peroxidase in the carotid body tissue with a concomittant decrease of the sinus nerve discharge. Also, in patients with chronic granulamatous disease based on mutated neutrophil NADPH-oxidase, oxygen sensing has not been found lacking (Bunn and Poyton, 1996), and NADPH-oxidase knockout mice had normal pulmonary arterial circulation response to hypoxia (K. Weir, personal communcation). But it is questionable whether the mutations of the neutrophil NADPH-oxidase leading to the disease or being characteristic for the knock-out mice affect also the low out put form. Differences of the high and low out put form with respect to the protein sequence and the protein concentration of the single components ($p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, rac) has been described (for more detailed information see Cool et al., 1998) To identify mitochondrial and non-mitochondrial cytochromes by light absorption photometry carotid bodies nominal free of hemoglobin have been used. Fig. 1b shows a record of chemoreceptor nervous activity exposed to a severe hypoxia for about 4 minutes stabilizing at lower discharge levels after a transient peak in spite of a continuous stimulus. The peak discharge rate occurred ahead of reduction of cytochromes. This is presumably because initially cytochrome reduction was small. With time, as the reduction of cytochromes becomes stronger, the chemosensory activity declines, as if the inhi-

bition of activity was associated with cytochrome reduction. Thus, excitation of chemoreceptors occurred at a time where no reduction of cytochromes can be seen. However, chemoreceptor inhibition occurred when cytochromes became reduced indicating the redox state of the carotid body tissue as an additional factor contributing to the non-linear interaction between oxygen and carotid body discharge. Compared to hemoglobin an about 5 times higher sensitivity was needed to record the cytochromes. This indicates by comparing the two records of Fig. 1a and 1b that hemoglobin depending on the intravascular concentration either makes cytochrome redox changes invisible for absorption photometry or contaminates the recorded spectra. Great care has to be taken therefore to rinse carotid body tissue free of red cells and to control the recorded cytochrome spectra for their composition by deconvolution. The recorded N_2 versus aerobic steady state spectra in Fig. 1b show clearly the development of absorption peaks at 550 nm and 602 nm and a shoulder at about 560 nm with the highest degree of optical density changes at the end of the hypoxic period. The composition of different mitochondrial and non-mitochondrial cytochromes forming these spectra is shown in Fig. 1c. Difference spectra of various isolated cytochromes have been used for this purpose to identify in a deconvolution procedure (Ehleben et al., 1997; Porwol et al., 1998) the peaks and the shoulder as well as to fit the experimental curve by a superposition curve. The curves of Fig. 1c marked with different symbols correspond to redox difference spectra of isolated mitochondrial cytochrome c peaking at 550 nm, b_{563} peaking at 563 nm and aa_3 peaking at 605 nm (Chance et al., 1975; Heinrich, 1981) as well as to the non-mitochondrial cytochrome b_{558} peaking at 558 nm as described for the NADPH oxidase in neutrophils (Thelen et al., 1993). These cytochromes have been shown by photometry and immunohistochemistry to be present in the carotid body tissue (Acker and Xue, 1995; Kummer and Acker, 1995). The amplitude of the optical density for the spectra of the isolated cytochromes was varied to fit the experimental curve (solid line) as close as possible with a superposition curve (open circles). The peaks at 550 nm and 602 nm of the experimental curve are clearly related to isolated cytochrome c and aa_3 spectra. The peak of isolated cytochrome aa_3 is shifted to 605 nm due to its reduction by cyanide (Heinrich, 1981). The shoulder seems to be composed of cytochrome b_{563} and b_{558}. The close fit of the experimental curve by the superposition curve as well as the significant difference of the light absorption peaks and troughs in Fig. 1a and 1b indicates a successful recording of redox kinetics of cytochromes in the carotid body tissue nominal free of hemoglobin. Deconvolution of the most reduced N_2 versus aerobic steady state spectrum as shown in Fig. 1c clearly indicates therefore the close relationship between reduction of mitochondrial and non-mitochondrial cytochromes and the stabilisation of chemoreceptor discharge at lower levels after a transient peak during superfusion with N_2 equilibrated medium. This is contrasted with studies on isolated type I cells showing that closing of P_{O_2} dependent potassium channels is linear between 160 and 20 Torr and accompanied by an increased intracellular calcium level and a subsequent transmitter release (Montoro et al., 1996). Also cyanide application in a dose up to 1mM is responded by type I cells with an increasing intracellular calcium level accompanied by a depression of the mitochondrial membrane potential indicating complete reduction (Duchen and Biscoe, 1992). The question was addressed therefore whether sinus chemoreceptor discharge generation underlies the control of an additional oxygen sensing cytochrome not detected by deconvolution revealing mitochondrial and non mitochondrial cytochromes probably closely related to redox control of transmitter release from type I cells. CO was used to for this purpose following studies by Wilson et al. (1994) revealing by action spectrum analysis cytchrome a_3 as an additional oxygen sensing heme protein.

CO as shown in Fig. 2a excites chemoreceptor discharge under conditions in a range between 60% to 10% O_2 in the dark. At 60% O_2 the effect of CO was least and gradually increased signifi-

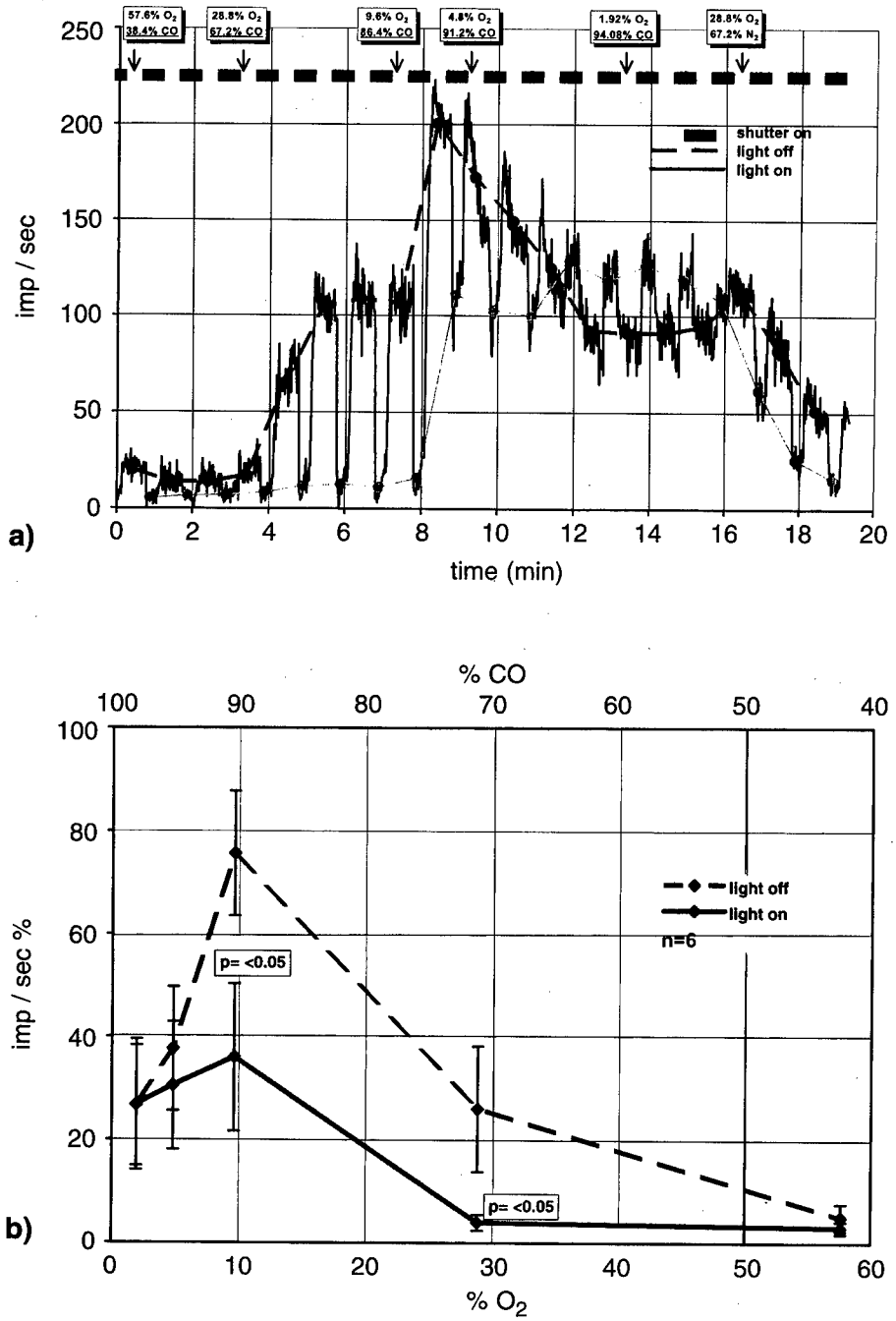

Fig. 2. Reaction of chemoreceptor discharge to superfusion of carotid body tissue with medium equilibrated with different gas mixtures of O_2/CO on a constant background of 4% CO_2. Photodissociation of the CO-heme binding by transillumination of carotid body leads under less hypoxic conditions to a decline and under hypoxic conditions to an increase of sinus nerve activity (a). Response curve between chemoreceptor discharge and O_2/CO equilibration of carotid body tissue summarized over six experiments (b). Dotted line indicates chemoreceptor discharge during association of the CO-heme binding and full line during photodissociation of the CO-heme binding. The optimal configuration of the heme for CO binding to maximally excite action potential generation seems to be reached at about 10% O_2.

cantly over 30% O_2 in the dark (minute 0 to 7). Light decreased the activity to the minimal levels. This means that at these high levels of superfusate P_{O_2} the carotid body in the dark reacts with CO as being stimulated by hypoxia. As superfusate oxygen content decreases to 10% O_2 (minute 7 to 9), the activity increased further in the dark but the activity also increased with light. Lahiri et al. (1995) have shown, with each photodissociation, an increase in oxygen consumption and possibly ATP production. At P_{O_2} of 10 Torr, however, the baseline discharge did not return because of hypoxic stimulation. Thereafter, at 5% O_2 and 2% O_2 (minute 9 to 17) the maximal discharge rate in the absence of light steadily decreased and then stabilized. In the course of time, light excited the chemoreceptors. This reversal of light effect is presumably due to depressed cellular (reflected in the diminished chemosensory activity) metabolism in the dark. Light would reverse the situation. Eventually, however, the two states would become equal, as we have seen in Fig. 2a. At this time O_2 was restored (30% O_2 and 70% CO, minute 17 to 19) and the normal chemoreceptor activity was resumed. Fig. 2b summarizes the response curves of chemoreceptor discharge to hypoxia in the presence (dotted line) of CO i.e. without transillumination and in the absence of CO i.e. with transillumination (full line). A statistical difference between the two curves is to be seen in an oxygen concentration range between 30% and 10% and a CO concentration range between 70% and 90% whereas the reversal of the light response under low oxygen conditions as shown in Fig. 2a is not statistical significant. Due to these characteristics the heme controlling chemoreceptor discharge seems to have at its maximal responsiveness an optimal configuration for CO-binding inducing an enhanced chemoreceptor discharge level. Below or above this optimal range configuration seems to be of less favorable heme configuration for CO-binding coinciding with a reduction or an oxidation of mitochondrial and non mitochondrial cytochromes. This redox dependent CO-heme binding as shown in Fig. 2 in combination with the very fast and prompt light effect hint in our view to a most local side of action. Type I cells are highly unlikely as candidates since CO application enhances dopamine release in a non photolabile manner probably indicating that CO leads to an unspecific general transmitter release without a particular involvement of a CO-heme interaction (Buerk et al., 1997). We speculate therefore that a heme controlling pore size of ion channels in the cell membrane of nerve endings might be mainly involved in the photolabile CO effects. The oxygen sensing property of nerve endings has been already studied by Mitchell et al. (1972) and Kiennecker et al. (1978) on the P_{O_2} sensitivity of the nervous discharge of neuromas formed by regeneration of the carotid sinus nerve. However, Zapata et al. (1976) observed that crushing the carotid sinus nerve at different distances from the carotid body produced recovery of chemosensory activity only when appositions between nerve fibers and glomus cells were reestablished enabling transmitter release into the synaptic cleft. These observations, however, do not necessarily exclude the assumed P_{O_2} sensitivity but might also hint to a maturation process of the nerve endings under the influence of the type I cells to develope these oxygen sensing properties.

4. Conclusions

Fig. 3 tries to hypothesize in a tentative model of nerve endings and type I cells the location of the different oxygen sensing cytochromes and their possible function during carotid body chemoreception. It is shown that P_{O_2} sensitive ion channels of type I cells might be controlled by the redox or thiol state of the cell which is mainly determined by ROS generated by mitochondrial and non-mitochondrial cytochromes (for review see Acker, 1994). ROS detected in type I cells (Cross et al., 1990) are reported to gate potassium channels (for review see Peers, 1997) and might therefore act as second messengers to control membrane potential and transmitter release of type I cells. Our observations of the fast and prompt photolabile chemoreceptor response to CO stimulation, however, are the first indications for an ion channel directly gated by a heme which has been already hypothesized for type I cells by

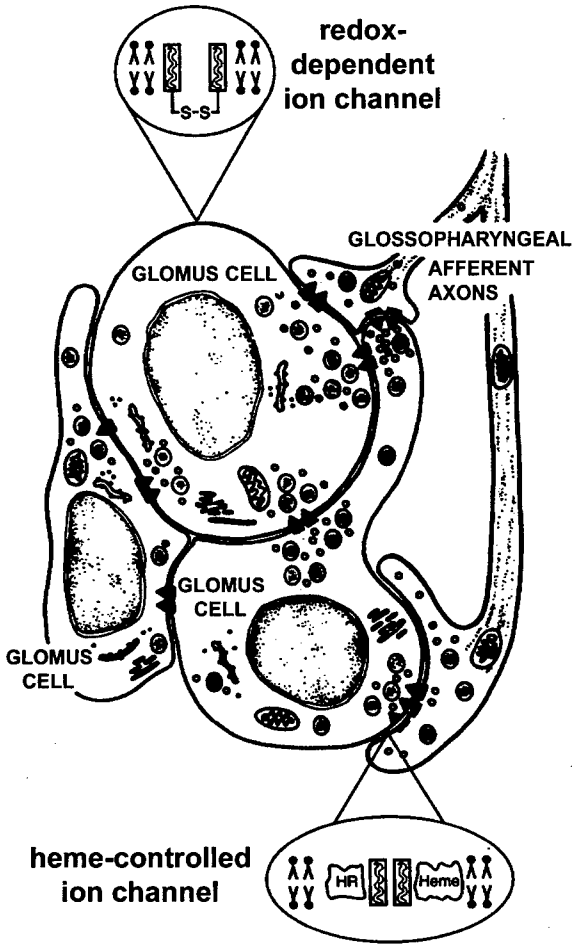

Fig. 3. Model of cellular composition of carotid body tissue (McDonald 1981) and the hypothesized location of ion channels either controlled by cellular redox status or by allosteric configuration of the attached heme in cooperation with a hormon receptor (HR) interacting with transmitters released from type I cells.

López-Barneo et al., (1993). We hypothesize that this ion channel is located at the nerve endings and determines in cooperation with transmitters released from type I cells the rate of action potential generation under hypoxia. As a main conclusion from this work we like to emphasize the necessity of studies both on type I cells as well as on nerve endings to understand more profoundly basic mechanisms of carotid body chemoreceptor discharge responding to hypoxia.

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Background leak K^+-currents and oxygen sensing in carotid body type 1 cells

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Abstract

One model of oxygen sensing by the carotid body is that hypoxia depolarises type 1 cells leading to voltage-gated calcium entry and the secretion of neurotransmitters which then excite afferent nerves. This paper revues the mechanisms responsible for the membrane depolarisation in response to hypoxia. It concludes that depolarisation is caused not through the inhibition of calcium activated or delayed rectifier K^+-channels but through the inhibition of an entirely new type of background K^+-channel. This channel lacks sensitivity to the classical K^+-channel inhibitors TEA and 4-AP. New evidence does however reveal that background K^+-channels in the type 1 cell can be inhibited by Ba^{2+} and that Ba^{2+} depolarises isolated type 1 cells. Intriguingly, Ba^{2+} is the only K^+-channel inhibitor thus far reported to stimulate the carotid body. These studies therefore support the hypothesis that depolarisation of the type 1 cell is an integral part of the oxygen sensing pathway in the carotid body.

Keywords: Oxygen sensing; Neurotransmitters; Depolarisation; Carotid body

1. Introduction

The current model of chemoreception in the carotid body is that stimuli, in the form of hypoxia or acidosis, are detected by specialised sensory cells (type 1 cells) which then signal via the secretion of neurotransmitters to afferent nerve endings. The sequence of events involved in the transduction of a hypoxic stimulus into a neurosecretory event is not fully understood, there is however good evidence that the latter stages of this pathway follow a familiar theme. Chemostimuli, including hypoxia, acidosis and some metabolic poisons, cause a membrane depolarisation which initiates electrical activity and calcium entry via voltage gated calcium channels (Buckler and Vaughan-Jones, 1994a,b, 1998; Montoro et

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al., 1996). The ensuing rapid rise of intracellular calcium then promotes the secretion of neurotransmitters via exocytosis (González et al., 1992; Ureña et al., 1994; Montoro et al., 1996). The principle objective of this revue is to consider the immediate cause of this depolarising receptor potential (see Fig. 1). At present, the ion channels responsible for the cells electrophysiological response to hypoxia represent the last identifiable step in the transduction of hypoxic stimuli, what lies upstream of these channels is unknown.

2. Methods

2.1. Cell isolation

Experiments were performed on type 1 cells enzymically isolated from the carotid bodies of neonatal rats (11–16 days old). The cell isolation

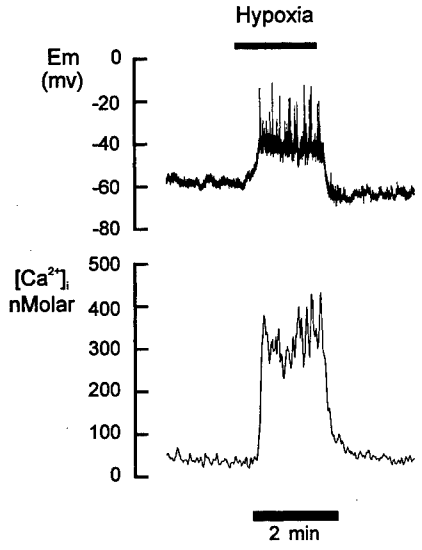

Fig. 1. Hypoxia depolarises rat type 1 cells. Simultaneous recording of membrane potential and intracellular calcium concentration in a single isolated rat type 1 Membrane potential was measured using the perforated patch whole cell recording technique. Intracellular calcium was measured using Indo-1. Hypoxia was induced by bubbling solutions with 95% N_2 = 5% CO_2 (P_{O_2} ≈ 5 torr). Note the rapid depolarisation of the resting membrane potential which is followed by some degree of electrical activity. Electrical activity is often weak in rat cells which reflects the lack of voltage-gated Na^+-channels.

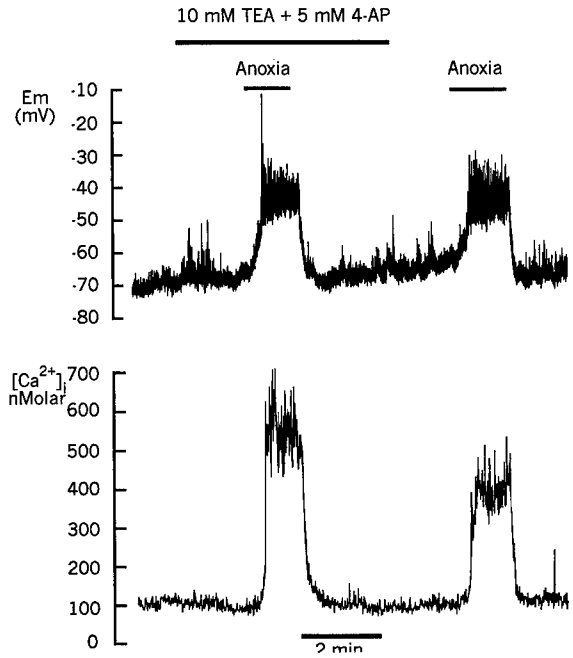

Fig. 2. Effects of K^+-channel inhibitors on membrane potential and intracellular $[Ca^{2+}]_i$. Effects of 10 mM TEA and 5 mM 4-AP upon membrane potential and intracellular calcium in a neonatal rat type 1 cell. Note that the combination of these two K^+-channel inhibitors has no effect upon resting membrane potential (or $[Ca^{2+}]_i$). In contrast, the cell depolarises rapidly in response to an anoxic stimulus (and $[Ca^{2+}]_i$ rises). Thus inhibition of calcium activated K^+-channels, or K_{O_2}-channels cannot alone account for the depolarising response to hypoxia. (Figure from Buckler, 1997)

procedure has been described in detail elsewhere (Buckler and Vaughan-Jones, 1993). Briefly, rat pups were anaesthetized with 4% fluothane in oxygen and the carotid bodies quickly excised and placed in ice cold saline. The carotid bodies were then dissociated in a phosphate buffered saline containing collagenase and trypsin. Isolated cells were plated out onto glass coverslips coated with poly-D-lysine and maintained in Hams F-12 culture medium (supplemented with 10% heat inactivated foetal calf serum 100 IU/ml penicillin, streptomycin 100 μg/ml and insulin 84 U/l) for 4–12 h until use.

2.2. Measurement of intracellular calcium

Cells were loaded with Indo-1 by incubation in

a solution of 2.5 μM Indo-1-AM in culture medium at room temperature for 1 h (Indo-AM was added from a 1 mM stock solution in DMSO). Indo-1 fluorescence was excited at 340 ± 5 nm and measured at 405 ± 16 and 495 ± 10 nm

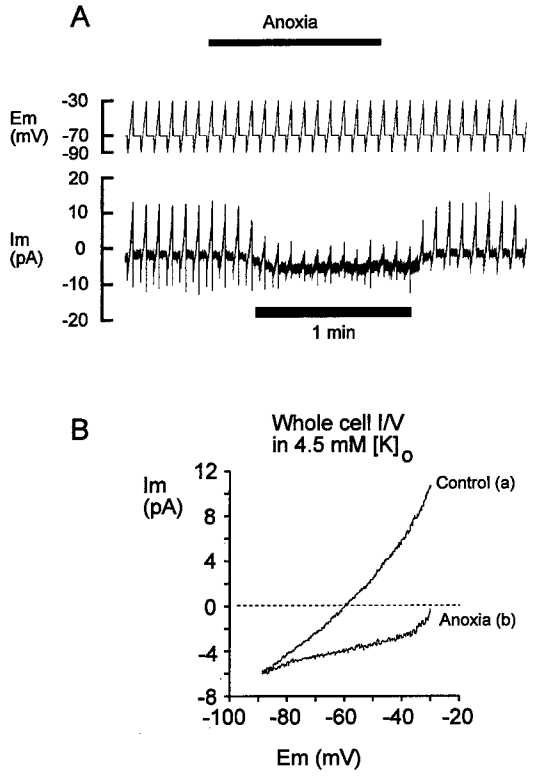

Fig. 3. Effects of hypoxia upon resting membrane currents. A. Recording of membrane current in a rat type 1 cell voltage-clamped at a holding potential of −70 mV and subject to periodic (0.2 Hz) voltage ramps from −90 to −30 mV (2 s duration). Note the inward (downward/depolarising) shift in holding current and the diminution of the ramp current during exposure of the cell to anoxia media. B. Mean current voltage-relations obtained from voltage ramps under both control and anoxic conditions for the cell in A. Note that under control conditions the current voltage relation transects the zero current axis at −60 mV. This zero current potential represents this cells resting membrane potential under control conditions. Under anoxic conditions there is no zero current potential (although it is very close to 0 at −30 mV) indicating that there is no longer a stable resting membrane potential in this range (i.e. the cell would have depolarised +ve to −30 mV had it not been held in voltage clamp). Note also the marked decrease in the slope of the current–voltage relation under anoxia conditions. This demonstrates the very marked decrease in resting membrane conductance that occurs in anoxia.

(using photomultiplier tubes). The calibration constants, R_{min}, R_{max}, and $F495_{free/bound}$ for Indo-1 were obtained from an in situ calibration protocol using ionomycin in a separate group of cells, Kd was assumed to be 250 nM (see Buckler and Vaughan-Jones, 1993).

2.3. Electrophysiology

All voltage and current-clamp recordings were performed using the perforated patch whole cell recording technique. The following filling solution was used for experiments depicted in Figs. 1–4; K_2SO_4 70, KCl 30, $MgCl_2$ 2, EGTA 1, HEPES 10; pH adjusted to 7.2–7.3 with NaOH. For experiments depicted in Figs. 5 and 6 the filling solution comprised K_2SO_4 55, KCl 30, $MgCl_2$ 2, EGTA 1, HEPES 20; pH adjusted to 7.2–7.3 with NaOH. Filling solutions also contained 240 μg/ml Amphotericin B (added from a stock solution of 60 mg/ml in di-methyl-sulfoxide). For further details of electrophysiological recording techniques, and selection criteria for successful recordings see Buckler (1997).

2.4. Solutions

The standard HCO_3^--buffered saline contained (in mM) NaCl 117, KCl 4.5, $NaHCO_3$ 23, $MgCl_2$ 1.0, $CaCl_2$ 2.5, glucose 11 and was equilibrated with 5% CO_2/95% air, pH 7.4–7.45. Hypoxic solutions were equilibrated with 5%CO_2 /95% N_2 and had a P_{O_2} of around 5 torr (measured in the experimental chamber). Anoxic solutions were also equilibrated with 5% CO_2/95% N_2 and contained 500 μM $Na_2S_2O_4$. All experiments were conducted at 33–37°C.

3. Results and discussion

3.1. Role of voltage-gated and Ca^{2+}-dependent K^+-channels

A number of electrophysiological studies of the carotid body have revealed that hypoxia has the ability to inhibit several different types of potassium channel in type 1 cells (Delpiano and Hes-

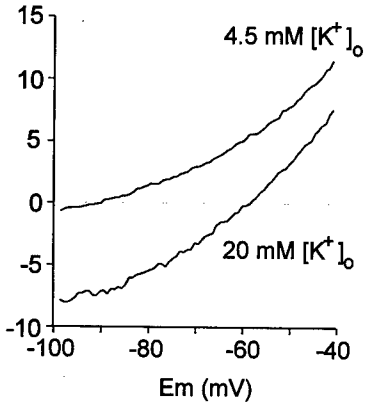

Fig. 4. Oxygen sensitive resting K^+-currents. Oxygen sensitive currents active around the resting membrane potential. Current–voltage relations were obtained using a ramp clamp protocol similar to that depicted in Fig. 3. Oxygen sensitive current was determined by subtracting the current obtained under anoxic conditions from that obtained under control conditions. Note shift in the reversal potential of the current–voltage relationship with change in extracellular $[K^+]$, indicating that the oxygen sensitive current is largely carried by K^+-ions (figure from Buckler, 1997)

cheler, 1989; López-López et al., 1989; Peers, 1990; Stea and Nurse, 1991). In particular hypoxia is reported to inhibit a delayed rectifier type K^+-channel in rabbit type 1 cells (the K_{O_2}-channel; López-López et al., 1989; Ganfornina and López-Barneo, 1992) and a large conductance calcium activated K^+-channel in rat type 1 cells (Peers, 1990; Wyatt and Peers, 1995). The precise role of these channels however is still far from clear.

It has been proposed that rabbit type 1 cells generate spontaneous rhythmic electrical activity and that the (hypoxic) inhibition of the K_{O_2} channel leads to a more rapidly depolarising pacemaker potential which increases firing frequency (López-Barneo et al., 1993). Doubt has, however, been expressed as to whether the biophysical properties of the K_{O_2}-channel would actually allow it to act as a pacemaker current since it only activates at potentials positive to -40 (e.g. López-López et al., 1993). Perhaps more importantly the relevance of this pacemaker model must itself be questioned in view of a recent report that the original recordings of pacemaker activity were unstable (Montoro et al., 1996). Indeed, in this latter work (Montoro et al., 1996), indirect recordings of electrical activity under normoxic conditions show only occasional action potentials every few seconds as opposed to the 10– 20 Hz firing frequency originally described (López-López et al., 1989; Ureña et al., 1989; López-Barneo et al., 1993). This observation makes it even more difficult to envisage how the inhibition of a delayed rectifier could increase action potential frequency in response to hypoxia.

In the rat type 1 cell it was proposed that the inhibition of a calcium activated K^+-current would cause a membrane depolarisation. Rat type 1 cells however have stable resting membrane potentials of around -50 mV (e.g. Fig. 1 and Buckler and Vaughan-Jones, 1994a,b), whereas the threshold for activation of the K_{Ca} channel is positive to -40 mV. It is therefore unlikely that K_{Ca}-channel activity would contribute significantly to the resting membrane potential of type 1 cells polarised to -50 mV (or more) with low levels of internal calcium (typically around 100 nM).

Although the above criticisms are theoretical, there is growing evidence to support the view that the inhibition of these two types of K^+-channel alone cannot account for the response to hypoxia in isolated cells or in the intact organ. Many studies have now shown that pharmacological inhibitors of K_{O_2} and/or K_{Ca}-channels do not excite neural discharge or secretion in whole carotid body preparations (Doyle and Donnelly, 1994; Cheng and Donnelly, 1995; Pepper et al., 1995; Donnelly, 1997; Osanai et al., 1997; Lahiri et al., 1998; Roy et al., 1998). Equally inhibitors of both the K_{O_2} channel (TEA and 4-AP) and K_{Ca}- channels (TEA and charybdotoxin), fail to increase $[Ca^{2+}]_i$ in isolated rat type 1 cells (Buckler, 1997). Indeed even a combination of 10 mM TEA plus 5 mM 4-AP failed to depolarise isolated rat type 1 cells (Fig. 2). Thus it would seem that the theoretical concerns are justified, the inhibition of K_{Ca} or K_{O_2} channels alone is not sufficient to evoke a chemoresponse either in isolated type 1 cells or in the intact organ.

3.2. Oxygen sensitive background/leak K^+-currents

In view of the above considerations the cause of the hypoxic membrane depolarisation has been directly investigated using the perforated patch whole cell voltage clamp technique. When rat type 1 cells are voltage-clamped at −70 mV the application of an anoxic stimulus evokes an inward shift in holding current of a few pA (Fig. 3A). At the same time there is a marked reduction in the resting membrane conductance (measured using voltage ramps Fig. 3). Under control conditions, the resting membrane conductance of rat type 1 cells is about 320 pS. This falls substantially in the presence of anoxia to around 130 pS (Buckler, 1997). These changes in the electrical properties of the type 1 cell membrane are the cause of the depolarisation in response to anoxia. Note that the control current–voltage plot (Fig. 3) shows a zero current potential of around −60 mV. This represents the resting membrane potential for this cell under control conditions. In the presence of anoxia however there is no zero current potential thus there is no stable resting membrane potential in this range (although I_m approaches 0 at −30 mV). Typically, in current clamp recordings, membrane potential depolarises to > −40 mV but is often accompanied by electrical activity such that there may be no stable resting membrane potential (see Fig. 1). What determines the frequency of electrical activity and

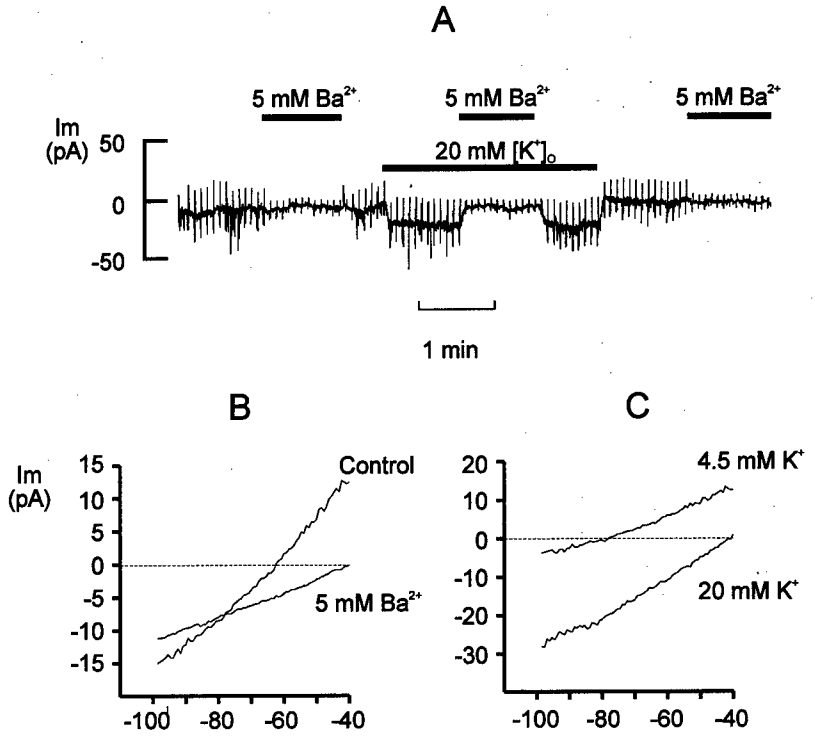

Fig. 5. Effects of barium on resting membrane conductances. A original recording of membrane current in a neonatal rat type 1 cell voltage clamped at a holding potential of −70 mV and subject to repetitive voltage ramps (0.2 Hz) from −100 to −40 mV (1 s duration; voltage record not shown). B Whole cell current voltage relation obtained from experiment depicted in A in normal extracellular $[K^+]$ in the absence (control) and presence of 5 mM Ba^{2+}. Note that Ba^{2+} decreases membrane conductance and shifts the zero current potential to a more positive value. C Ba^{2+}-sensitive current voltage relation (control-Ba^{2+}) in 4.5 mM extracellular $[K^+]$ and in 20 mM $[K^+]_o$. Shift in reversal potential of Ba^{2+} sensitive current with change in $[K^+]_o$ indicates that the Ba^{2+} sensitive current is a K^+-current (reversal potentials = −80 and −39 mV; E_K = −88 and −50 mV at $[K^+]_o$ = 4.5 and 20 mM, respectively).

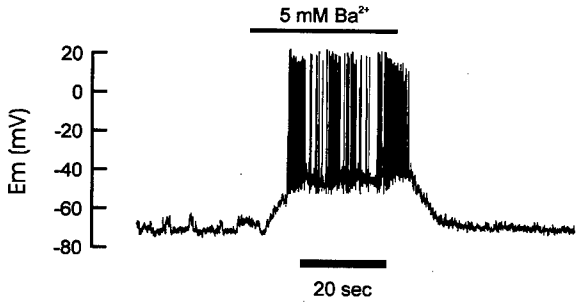

Fig. 6. Effects of Ba^{2+} on membrane potential. Recording of membrane potential in a neonatal rat type 1 cell (perforated patch). Note rapid depolarisation and electrical activity in response to the application of 5 mM Ba^{2+}.

membrane potential in the presence of hypoxia has not yet been determined.

The difference between the two current–voltage (I–V) curves obtained under control and anoxic conditions represents the oxygen sensitive current. What is this current? I have measured this current at varying levels of extracellular K^+ and have found a near Nernstian shift in its reversal potential upon raising $[K]_o$ from 4.5 to 20 mM (Fig. 4; $E_{rev} = -90$ and -58 mV; $E_K = -96$ and -57 mV, respectively). These observations indicate that this oxygen sensitive current is indeed a K^+-current. Further characterisation of this current has revealed that it is quite distinct from either the K_{O_2} or K_{Ca} channels in that it is not inhibited by 10 mM TEA or 5 mM 4-AP (Buckler, 1997). This current is referred to as a background or leak current because it appears to lack any intrinsic voltage sensitivity and is time independent (Buckler, 1997). Its most important attribute, in the context of chemoreception, however is that it is active at, and plays a role in maintaining, the resting membrane potential. The inhibition of this background K^+-current by hypoxia is, therefore, a key factor in causing membrane depolarisation.

3.3. Barium inhibits background K^+-conductance

I have sought further evidence that the inhibition of a background K^+-channel could account for the depolarisation seen in hypoxia. Although we have not, as yet, found any specific blockers of the background K^+-channel, barium at sufficiently high concentrations will inhibit background K^+-current. Fig. 5(A,B) shows that 5 mM Ba reduces resting membrane conductance in rat type 1 cells. The reversal potential of the Ba-sensitive current (Fig. 5C) shifts by about 40 mV as extracellular K^+ is increased from 4.5 to 20 mM indicating that the Ba^{2+}-sensitive current is a K^+-current. The extent to which 5 mM Ba^{2+} inhibits the resting K^+-conductance can be estimated from the difference in holding current between 4.5 and 20 mM K^+ under control conditions (19.2 pA) versus 5 mM Ba^{2+} (3.0 pA) and is around 85%. Ba^{2+} was also found to substantially inhibit the oxygen sensitive background conductance in type 1 cells (not shown). Thus the Ba^{2+}-sensitive background K^+-current includes the O_2-sensitive background K^+-current. At present, however, we cannot tell whether Ba^{2+} is specific for only oxygen-sensitive background K^+-channels (there may well be other Ba^{2+}-sensitive background K^+-channels that are not O_2-sensitive).

If the hypothesis that background K^+-channel inhibition causes membrane depolarisation is correct, then Ba^{2+} should also depolarise type 1 cells. Fig. 6 shows that this is indeed the case, 5 mM Ba^{2+} causes a marked depolarisation in rat type 1 cells (mean $E_m = -59.2 \pm 4.6$ mV control; -34.4 ± 2.3 mV in 5 mM Ba^{2+}; $n = 4$). It is also notable that 3 mM Ba^{2+} induces an intense excitation of neural discharge in the rat carotid body (Donnelly, 1997). Whilst barium is perhaps not the ideal pharmacological tool with which to prove a role for a specific K^+-channel, these results do support the principle that inhibition of some forms of K^+-channel can lead to excitation both of isolated cells and of the intact carotid body.

3.4. Modulation of background K^+-channels

There is now evidence that the same, or a very similar, background K^+-channel may be modulated by a number of other chemostimuli. We have previously shown that acidosis reduces

resting membrane conductance and that the reversal potential of this current is around −75 mV (Buckler and Vaughan-Jones, 1994b). This strongly suggests that acidosis also inhibits a background K^+-conductance. Recent studies on the effects of metabolic poisons have shown that the uncouplers DNP and FCCP inhibit a background K^+-current (Buckler and Vaughan-Jones, 1998) as does cyanide (unpublished). Thus the transduction pathways for a number of potent chemostimuli appear to converge upon a common target protein the background K^+-channel. This observation raises the question as to whether background K^+-channels might themselves be modulated by some common mechanism for all these stimuli. It would be tempting to suggest that an intracellular acidosis might be a common final pathway. Studies in this laboratory however indicate that hypoxia has little effect upon pH_i and, whilst uncouplers do generate a small fall in pH_i, the response to DNP is not antagonised by intracellular alkalinisation (Buckler and Vaughan-Jones, 1998). Thus it seems that changes in pH are not responsible for the sensing of hypoxia or the response to metabolic inhibitors.

The fact that mitochondrial inhibitors act in a manner remarkably similar to hypoxia (i.e. inhibition of background K^+-current, membrane depolarisation and voltage-gated calcium entry, Buckler and Vaughan-Jones, 1998) does, however, resurrect the possibility that mitochondrial metabolism may play a role in oxygen sensing in the type 1 cell. In this context it is important to note that the response to uncouplers is fast in onset with ionic currents responding within seconds to the application of uncoupler (Buckler and Vaughan-Jones, 1998), indicating that there is a rapid signalling pathway linking changes in mitochondrial respiration to ion channel activity. Moreover, the data of Duchen and Biscoe (1992) suggests that mitochondrial metabolism in the type 1 cell has a similar oxygen sensitivity to the background K^+-conductance, ie with changes in mitochondrial potential (Duchen and Biscoe, 1992), and inhibition of background K^+-conductance (Buckler, 1997) beginning as P_{O_2} falls below 40 torr. Thus the mitochondrion could in principle act as an effective detector of hypoxic stimuli for the purposes of regulating background K^+-currents. The challenge which faces us now is to determine the nature of this mitochondrial signalling pathway and to establish whether it is actually involved in oxygen sensing.

3.5. Identity of oxygen-sensitive background K-channels

The biophysical and pharmacological studies we have thus far performed upon the background K^+-current, provide some clues as to the possible identity of the channels involved. The lack of intrinsic voltage sensitivity and the resistance to block by TEA and 4-AP strongly suggests that these background channels do not belong to either the outward rectifying K_v channel or the inwardly rectifying, K_{ir} channel families. A third family of mammalian K^+-channels have however recently been cloned (Fink et al., 1996, 1998; Lesage et al., 1996; Duprat et al., 1997). These channels have a structure quite distinct from K_V and K_{ir} type channels in that they have two pore forming loops per subunit instead of one. When expressed these subunits form active channels which are resistant to block by TEA and 4-AP. Of the four channels cloned and expressed to date, two show some weak voltage sensitivity, TWIK and TREK (Fink et al., 1996; Lesage et al., 1996). The other two TASK and TRAAK show no intrinsic voltage sensitivity, only GHK type rectification in asymmetric K^+-ion gradients (Duprat et al., 1997; Fink et al., 1998; Kim et al., 1998; Leonoudakis et al., 1998). It is these latter two channels which display greatest similarity to the background K^+-current in the type 1 cell. Of particular interest is TASK since this channel is also very pH sensitive (Duprat et al., 1997; Leonoudakis et al., 1998). In preliminary studies we have identified a candidate channel in cell attached patches from type 1 cells which is active at the resting membrane potential, is inhibited by hypoxia and has a single channel conductance of 14 pS (Williams and Buckler, 1998). This compares favourably with the single channel conductance of 16 pS reported for TASK. Definitive identification however will have to await further studies of the properties of this new type 1 cell K^+-channel.

3.6. Re-evaluation of the role of other O_2-sensitive channels

Do the above studies mean that the modulation of delayed rectifier or Ca^{2+}-activated K^+-channels by hypoxia are irrelevant to oxygen chemoreception, or that they are 'an epiphenomenon' (Lahiri et al., 1998). I believe not, only that it is important to establish the context in which they influence the response to hypoxia. Consider the following hypothetical model. Under normoxic conditions a stable membrane potential of -70 to -50 mV is maintained through the balance of electro-diffusive ionic fluxes through background K^+-channels, and presumably others i.e. Na^+ and Cl^- channels, plus some contribution from electrogenic transporters. With hypoxia, background K^+-channels are inhibited and the membrane starts to depolarise. As the membrane voltage becomes positive to about -40 mV other ionic channels will begin to come into play. Activation of voltage-gated Ca^{2+}-channels will promote further depolarisation, possibly leading to the generation of an action potential. The amplitude and duration of this action potential will depend upon the magnitude, and inactivation properties of these voltage-gated Ca^{2+}-currents and the magnitude and rate of activation of delayed rectifier K^+-channels. With electrical activity, and possibly also through the receptor potential itself, $[Ca^{2+}]_i$ will rise activating Ca^{2+}-dependent K^+-channels. Activation of K_{Ca}-channels will oppose the generation of action potentials and may even begin to repolarise the membrane slightly. With background K^+-channels remaining inhibited however the membrane could not fully repolarise since this would lead to a cessation of Ca^{2+}-influx a fall in $[Ca^{2+}]_i$ deactivation of Ca^{2+}-activated K^+-channels and depolarisation again. With sustained stimulation, or with strong stimuli action potentials may eventually subside and a new stable, but depolarised, potential will be established in which steady state inward Ca^{2+}-currents balance steady state outward K^+-currents through delayed rectifiers and K_{Ca}-channels (plus a small contribution from remaining background conductances).

The purpose of the above discussion is to illustrate that although voltage-gated and or Ca^{2+}-dependent K^+-channels do not play a role in determining the resting potential under normoxic conditions, there is every probability that they will be important in determining electrical events in depolarised cells. Direct modulation of voltage-gated and/or Ca^{2+}-activated K^+-channels by hypoxia could, therefore, still play an important role in defining the $[Ca^{2+}]_i$ response to hypoxia. Clearly there is much more careful electrophysiology to be done before we can really claim to understand which channels do what and how in the type 1 cell. A role for the Ca^{2+}-dependent K^+-channel in determining the magnitude of the response to a hypoxic stimulus is however indicated by studies in intact rat carotid bodies which show that charybdotoxin (a K_{Ca}-channel inhibitor) can potentiate the response to hypoxia even though it alone does not excite the chemoreceptor (Pepper et al., 1995).

3.7. Future studies

Aside from the above considerations regarding the development of a more precise model of electrical signalling in type 1 cells, perhaps the most pressing issue is that of the oxygen sensor itself. As outlined in the introduction, oxygen sensitive ion channels represent the last known entity in the oxygen sensing pathway in type 1 cells. It is therefore highly probable that much effort will now focus upon attempts to identify the next step in the pathway through a combination of biochemical and structural/molecular studies upon these channels.

Acknowledgements

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Acetylcholine contributes to hypoxic chemotransmission in co-cultures of rat type 1 cells and petrosal neurons

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Abstract

The neurotransmitter mechanisms that mediate chemosensory transmission in the mammalian carotid body (CB), i.e. the primary arterial P_{O_2} detector, are controversial. Given the inherent difficulty of recording from afferent terminals in situ, the authors have adopted an alternative approach based on co-culture of dissociated CB receptor (type 1) cell clusters and petrosal neurons (PN) from 8–14-day-old rat pups. Electrophysiological, perforated patch recordings from petrosal somas, juxtaposed to type 1 clusters, revealed the development of a high incidence of functional 'synapses' in vitro. Recent evidence has strengthened the case for acetylcholine (ACh) as a co-released transmitter: (i) cultured type 1 cells express several cholinergic markers including the vesicular ACh transporter (VAChT), intracellular acetylcholinesterase (AChE), and occasional clear cored vesicles ($\approx$ 50 nm diameter); (ii) the frequency of spontaneous synaptic activity, as well as the hypoxia-induced depolarization recorded in 'juxtaposed' PN in co-culture, were partially suppressed by the nicotinic ACh receptor (nAChR) blocker, mecamylamine (2 μM); (iii) consistent with the presence of extracellular AChE, ACh-mediated membrane noise in type 1 cells as well as the hypoxia-evoked PN response in co-culture were potentiated in a few cases by the AChE inhibitor, eserine (100 μM). Thus, since many PN and type 1 cells express mecamylamine-sensitive nAChR, released ACh may act presynaptically on type 1 cell autoreceptors and/or postsynaptically on petrosal terminals. Other CB transmitter candidates (e.g. 5-HT and ATP) were found to excite PN, though their potential role as co-released sensory transmitters requires further investigation.

Keywords: Chemotransmission; Co-culture; Glomus cell; Petrosal neurons; Hypoxia

1. Introduction

The synaptic mechanisms that mediate the carotid body (CB) chemoexcitatory response to hypoxia have been a controversial subject for over

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50 years. While there appears to be a consensus that CB type 1 cells are the actual sensory transducers during hypoxaemia (González et al., 1994; López-Barneo, 1996, see however Sun and Reis, 1994), the nature of the excitatory transmitter(s) that translate type 1 cell depolarization into a train of action potentials in carotid sinus nerve (CSN) remains arguable. Supportive evidence for acetylcholine (ACh) as a major excitatory transmitter in CB chemosensory signalling emerged and accumulated since the 1930s (Schweitzer and Wright, 1938; Hollinshead and Sawyer, 1945; Eyzaguirre and Zapata, 1968). However, this idea was challenged in the intervening years (Douglas, 1954; Sampson, 1971; McQueen, 1977), only to undergo a recent resurgence (Fitzgerald et al., 1995, 1997; Zhong et al., 1997). Other neurotransmitter candidates, including dopamine and substance P, have also been proposed as mediators of CB chemoexcitation (Prabhakar et al., 1993; González et al., 1994; Prabhakar, 1994).

The most direct resolution to this problem requires the recording of synaptic events from sinus nerve terminals opposed to type 1 receptor cells in situ during hypoxic chemotransduction. This approach has met with limited success due to access difficulties, poor visibility, and unstable recording conditions (Hayashida et al., 1980). Alternative approaches which rely solely on recording of extracellular CSN activity suffer from the disadvantage that subthreshold synaptic events are not recorded, thereby precluding a clear interpretation of the mechanisms mediating chemical transmission (Katz, 1969). The authors have had recent success with a compromise approach, based on co-culture of dispersed rat CB type 1 cell clusters and dissociated petrosal ganglia (Zhong et al., 1997), which contain the cell bodies of chemoafferent CSN fibers (McDonald, 1981; Finley et al., 1992). In these co-cultures, subthreshold events can be recorded from a substantial proportion of neurons located close to a type 1 cell cluster, with which they appear to form de novo chemical synapses. Most importantly, at some of these complexes consisting of a type 1 cluster and a juxtaposed neuron, a hypoxic stimulus is transduced in the type 1 cells and the information relayed to the neuron as a depolarization and/or increased action potential frequency (Zhong et al., 1997).

Here, the potential usefulness of this co-culture preparation as a model system to aid resolution of the 'transmitter question' in the carotid body is reviewed, and additional evidence in support of ACh as an excitatory co-transmitter in chemosensory signalling is provided.

2. Methods

2.1. Culture techniques and electrophysiology

The procedures for obtaining cultures of dispersed type 1 cell clusters, with and without petrosal neurons (PN) from 8–14-day-old rat pups have been described in detail elsewhere (Nurse, 1987, 1990; Stea and Nurse, 1992; Zhong et al., 1997). Recordings of membrane potential under current clamp (zero current) conditions were carried out with the nystatin perforated-patch technique to minimize disturbance of the intracellular milieu, as previously described (Zhong and Nurse, 1997a; Zhong et al., 1997, 1999). All recordings were done at $\approx 35°C$ in medium buffered with bicarbonate/CO_2 and perfused at the rate of ≈ 2 ml min^{-1} (Zhong et al., 1997). The hypoxic stimulus (achieved by equilibrating the medium with 95% N_2/5% CO_2) was delivered via a rapid perfusion system (Zhong et al., 1997), and neurotransmitters were applied by rapid perfusion or by pressure ejection from a 'puffer' pipette (Zhong and Nurse, 1997a; Zhong et al., 1999). Eserine was obtained from Sigma (St. Louis, MO, USA) and mecamylamine from Research Biochemicals International (Natick, MA, USA).

2.2. Acetylcholinesterase cytochemistry and electron microscopy

The details of these procedures are identical to those previously described (Nurse, 1987; Nurse et al., 1993). To inhibit non-specific cholinesterase the cultures were pre-incubated with 10 μM tetraisopropyl-phosphoramide (iso-OMPA, Sigma). For electron microscopy, cultures were fixed in 1% glutaraldehyde and post-fixed with 2% OsO_4.

2.3. Immunofluorescence

The procedures for dual labelling co-cultures with antibodies against tyrosine hydroxylase (TH) and neurofilament protein (NF 68 kd), visualized with FITC- and Texas Red- conjugated secondary antibodies respectively, are described in detail elsewhere (Zhong et al., 1997). For localization of vesicular acetylcholine transporter (VAChT), cultures were fixed in 4% paraformaldehyde, and incubated overnight at 4°C with goat anti-VAChT antibody (1:300; Chemicon, El Segundo, CA, USA) in phosphate buffered saline (PBS) supplemented with 10% normal goat serum, 1% bovine serum albumin, and 0.05% Triton X-100. To aid visualization, cultures were washed 3 × in phosphate buffer, before incubation for 1 h with FITC-conjugated donkey anti-goat IgG (1:20 dilution; Jackson Immunoresearch Laboratories, West Grove, PA, USA). In control experiments, omission of the primary antibody abolished all staining. For visualization of VAChT staining, cultures were exposed to a photobleaching reagent before viewing in a Zeiss IM35 inverted microscope, equipped with epifluorescence.

3. Results

The most widely accepted markers for type 1 or glomus cells relate to their adrenergic phenotype, characterized by the expression of catecholaminergic enzymes (e.g. tyrosine hydroxylase), catecholamine histofluorescence, and the presence of large cytoplasmic dense cored vesicles (McDonald, 1981; González et al., 1994). However, these cells appear to express a broad transmitter repertoire, including several biogenic amines and neuropeptides (Wang et al., 1992; González et al., 1994; Prabhakar, 1994). The status of ACh is considered below since it is proposed to play a key role as a co-released transmitter during CB chemosensory processing.

3.1. Cholinergic markers in type 1 cells

There is abundant evidence that the CBs of several species contain and release ACh (Eyzaguirre and Zapata, 1968; González et al., 1994). Previous immunocytochemical studies provided evidence that type 1 cells are the sites of ACh synthesis, since the biosynthetic enzyme choline acetyltransferase (ChAT) was localized specifically to these cells in cat and rabbit CB in situ, where it was frequently co-expressed with amine-synthesizing enzymes (Wang et al., 1989, 1992). In addition, histochemical evidence indicated that the degradative enzyme, acetylcholinesterase (AChE), was localized to amine-positive type 1 cells in rat CB in situ (Korkala and Waris, 1977; Nurse, 1987). These studies, together with the autoradiographic demonstration of a high affinity uptake mechanism for choline (Wang et al., 1989), provide strong evidence that type 1 cells normally express a cholinergic phenotype in vivo.

Following isolation and growth for several weeks in dispersed cell culture, rat type 1 cell clusters retain several cholinergic properties, including the presence of cytochemically-detectable AChE, and occasional small clear cored vesicles ($\approx$ 50 nm) in the electron microscope (Nurse, 1987; Nurse et al., 1993). An example illustrating AChE staining in two type 1 cell clusters in a 18-day-old CB culture is shown in Fig. 1a. Examination of the AChE reaction product in the electron microscope revealed both an intracellular and extracellular localization associated with type 1 cell clusters (Nurse, 1987). As indicated in Fig. 1b,c, electron microscopic examination of these cultured cells also indicated the presence of clusters of clear cored vesicles (not unlike those seen at cholinergic synapses) near the junctional membranes of adjacent type 1 cells. These vesicles sometimes appeared to be associated with darkly stained dense bodies (Fig. 1b,c; open arrow heads), that resembled the presynaptic dense projections commonly seen at chemical synapses (McDonald, 1981). On occasion, clear vesicles were seen near coated vesicles and presynaptic dense bodies (e.g. Fig. 1b; central arrow head), or adjacent to a dense cored granule (Fig. 1c; arrow). The clear vesicles were about one-half the diameter of the dense cored granules, which average $\approx$ 100 nm in cultured type 1 cells (Nurse et al., 1993).

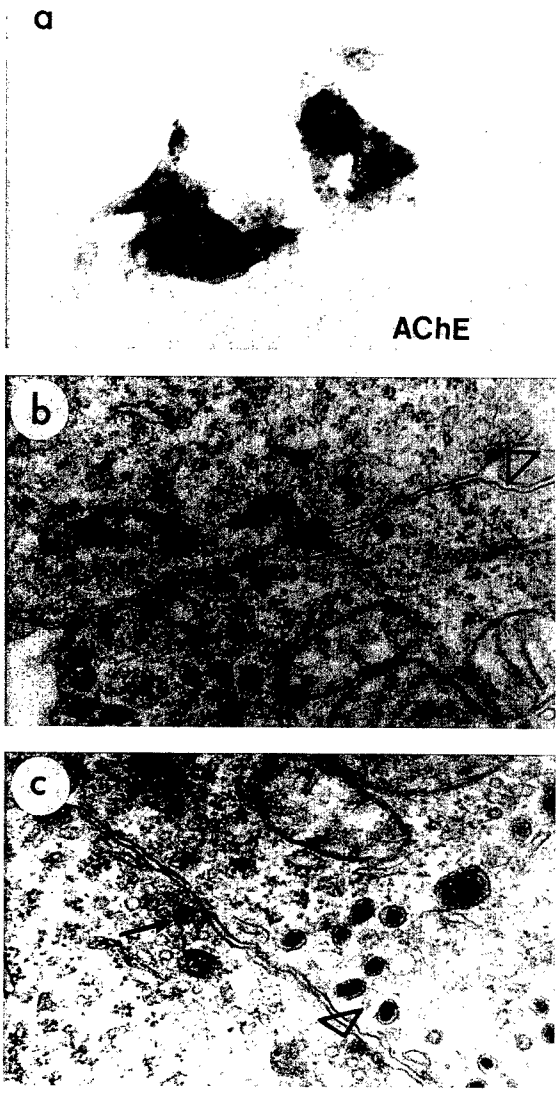

Fig. 1. Cholinesterase staining and electron micrographs of cultured rat type 1 cells. (a) The 18-day-old culture of dissociated carotid body cells was stained for AChE activity using a modification of the Karnovsky procedure (Nurse, 1987). Two type 1 cells clusters are positively stained for AChE reaction product; magnification $\sim 200\times$. (b, c) The fine structural features of junctional regions between neighboring type 1 cells in a 14 day-old culture are revealed by electron microscopy. Note the characteristic dense cored granules (≈ 100 nm in diameter) in type 1 cells, and the presence of clear cored vesicles adjacent to presynaptic dense projections indicated by open arrow heads in. In (b) the central arrow head also locates a coated vesicle to the left of the presynaptic density. In (c), the arrow indicates a dense cored vesicle surrounded by a cluster of clear cored vesicles. These clear cored vesicles are ≈ 50 nm in diameter and resemble those seen at cholinergic synapses.

In recent studies, positive immunostaining of cultured rat type 1 cells for the VAChT was found, a more recently characterized cholinergic marker that shares a common gene locus, and regulatory elements for gene transcription, with the biosynthetic enzyme ChAT (Eiden, 1998). An example from a 2-day-old culture of VAChT-positive type 1 cell clusters, visualized by FITC-immunofluorescence, is illustrated in Fig. 2a; background cells (not visible in the dark field micrograph) were unstained. Positive VAChT immunostaining was also seen in singly-isolated type 1 cells and doublets, and occurred in cultures as old as 17 days (not shown). In control experiments, staining was abolished when the primary antiserum was omitted.

3.2. Evidence for spontaneous acetylcholine release in rat carotid body cultures

The evidence described above indicates type 1 cells have the capacity for ACh synthesis, storage and degradation. A prerequisite for its function as a neurotransmitter is that it is released spontaneously and following physiological stimulation (Katz, 1969). Evidence was sought for spontaneous ACh release in type 1 cell cultures grown with or without PNs. To test for spontaneous release in cultures without neurons, the authors took advantage of the fact that rat type 1 cells express surface nicotinic ACh receptors (nAChR), sensitive to the ganglionic blocker, mecamylamine (Wyatt and Peers, 1993; Jackson and Nurse, 1998). An example is shown in Fig. 3a, where rapid perfusion of ACh (50 μM) during the period indicated by the lower horizontal bar caused type 1 cell depolarization (≈ 10 mV), which was abolished (reversibly) in the presence of 2 μM mecamylamine (middle trace). Thus, if ACh is released spontaneously at synaptic-like specializations between neighboring type 1 cells (Fig. 1b,c), inhibition of extracellular acetylcholinesterase (Fig. 1a) with 100 μM eserine might cause an increase in membrane noise due to prolonged autocrine and/or paracrine action of ACh. Evidence for this increase in membrane noise was obtained in four cases during current clamp recordings of resting membrane potential from

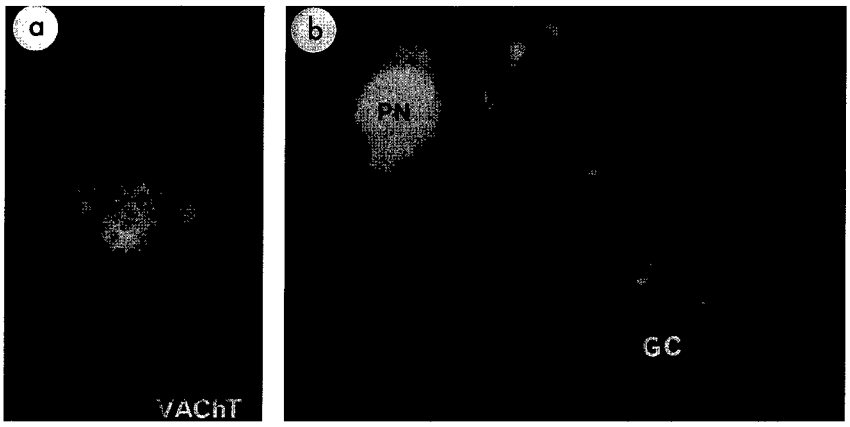

Fig. 2. Immunofluorescence staining of type 1 cell clusters grown with and without PNs. (a) Shows a small and large type 1 cell cluster from a 2-day-old culture immunostained with goat anti-vesicular acetylcholine transporter (VAChT) antibody, and visualized with a FITC-conjugated secondary antibody; background cells, not visible in the dark field micrograph, were unstained. (b) Shows a PN and a cluster of four type 1 or glomus cells (GC) from a co-culture that was immunostained with anti-NF (68 kDa) and anti-TH antibodies. The PN cell body was positive for both TH (visualized with a FITC-conjugated secondary antibody) and NF (visualized with a Texas Red-conjugated secondary antibody); the four GC were positive for TH only, and have a flattened appearance due to a longer time (21 days) in culture. Note the NF-positive processes that branch from the neuronal cell body, and the brightly-stained nerve endings which appear to terminate on the glomus cells, after only one day in co-culture. Magnification $\sim 150\times$ in (a) and $\sim 1000\times$ in (b).

type 1 cells; an example of the fluctuations in membrane potential before, during, and after 100 μM eserine is shown in Fig. 3b. In two of these cases, one of which is illustrated in Fig. 3c (traces from the same cell shown in Fig. 3b), it was confirmed that this increase in noise was due to activity of nAChR, since it was virtually eliminated when 2 μM mecamylamine was present together with eserine (middle traces in Fig. 3b,c).

3.3. Functional interactions in co-culture: evidence for spontaneous and hypoxia-evoked acetylcholine release

In order to study chemosensory transmission in the carotid body, a co-culture model was developed in which isolated PNs extend processes, some of which appear to form functional de novo connections with type 1 clusters (Zhong et al., 1997). An example of a co-culture immunostained for both TH (visualized with a FITC-conjugated secondary antibody) and neurofilament (68 kDa; visualized with a Texas Red-conjugated secondary antibody) is shown in Fig. 2b. In this dark field micrograph, the soma of the PN is positive for both NF and TH, whereas the adjacent four type 1 or glomus cells (GC) in the small cluster are positive for TH only. The long duration (≈ 3 weeks) in culture accounts for the somewhat flattened appearance of the four type 1 cells. Note several bright NF-positive endings from the PN appear to terminate on the type 1 cells after only 1 day in co-culture. Since carotid body chemoafferent neurons in the petrosal ganglia are known to express a dopaminergic (TH+) phenotype (Finley et al., 1992), it appears that at least some of these chemosensory neurons survive the culture conditions.

In these co-cultures, a high incidence ($\approx 40\%$) of functional interactions between type 1 clusters and PNs was detectable in CO_2/bicarbonate-buffered medium (Zhong et al., 1997). In these experiments, perforated patch recordings were obtained from 'selected' PNs that were juxtaposed to a type 1 cell cluster in order to facilitate monitoring of subthreshold events. In successful cases, spontaneous subthreshold events and/or spike activity could be detected in the juxtaposed neuron (Fig. 4a), and application of a hypoxic stimulus ($P_{O_2} \approx 5$ mmHg) resulted in membrane depolar-

ization (Fig. 4b,c), that was sometimes sufficient to trigger an increase in spike frequency (Zhong et al., 1997). Such responses were notably absent in the majority of >100 recordings from PNs cultured without type 1 cells (Zhong et al., 1997). To date, a small hypoxia-induced depolarization has been detected in only 5 PNs cultured without type 1 cells, but the low incidence prevented any further investigation. Their occurrence nevertheless, raised the possibility that contact with type 1 cells might enhance the expression of O_2-sensing properties in PNs (Sun and Reis, 1994). To exclude this possibility, and confirm that type 1 cells were the actual transducers, it was necessary to demonstrate that chemical synaptic transmission mediated hypoxic signalling in co-culture.

As shown in Fig. 4a, the spontaneous activity (spikes and subthreshold 'synaptic' potentials) recorded from juxtaposed PNs in co-culture was sensitive to 2 μM mecamylamine, suggesting it was due, at least in part, to spontaneous release of ACh acting on nAChR. The authors reached the same conclusion when another nicotinic ganglionic blocker, hexamethonium (100–200 μM), was used on similar co-cultures (see Zhong et al., 1997). Most important however, the hypoxia-evoked depolarization in co-cultured neurons was partially inhibited by 2 μM mecamylamine (Fig. 4b), consistent with the previous studies using 100–200 μM hexamethonium (Zhong et al., 1997). In the present experimental series, the mean ($\pm$ S.E.M.) hypoxia-induced depolarization in a group of 8 co-cultured neurons was 4.5 ± 0.82 mV before, 2.4 ± 0.45 mV during, and 4.33 ± 0.78 mV after, perfusion of the culture with 2 μM mecamylamine; this corresponds to $\approx 45\%$ inhibition of the response by the nicotinic blocker. Moreover, consistent with cholinergic transmission, the hypoxia-induced depolarization was potentiated in a few cases when AChE activity was inhibited with 100 μM eserine (Fig. 4c). These results, together with the demonstration that the spontaneous activity was also sensitive to reduction of the extracellular Ca^{2+}:Mg^{2+} ratio (Zhong

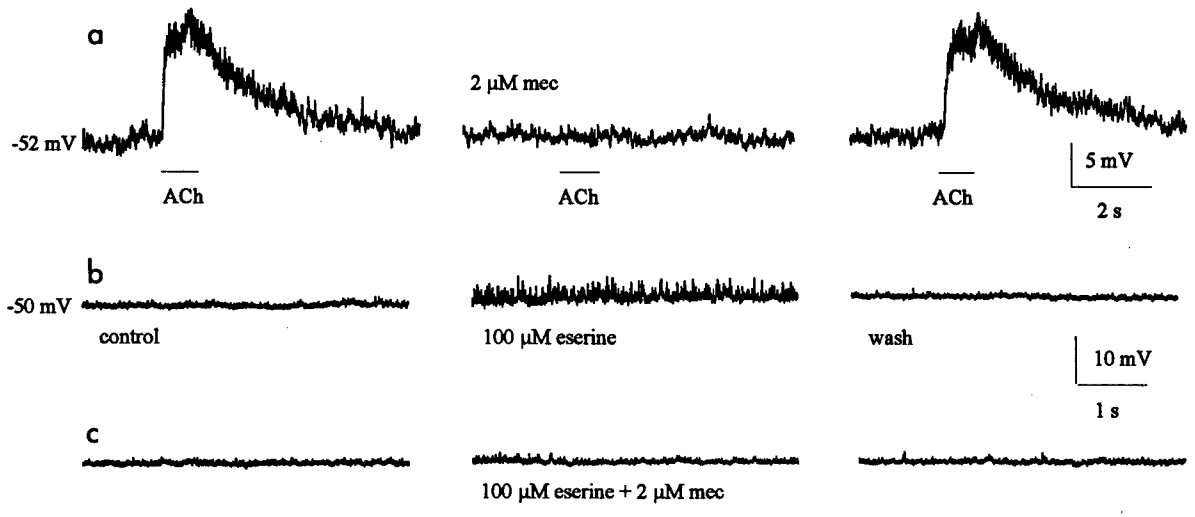

Fig. 3. Effects of ACh and AChE inhibitors on membrane potential of type 1 cells. Application of ACh (50 μM) by fast perfusion, during the period indicated by the lower horizontal bars in (a), caused membrane depolarization (left trace) recorded with the perforated-patch technique (in zero current mode). The depolarization was mediated via neuronal nicotinic AChR since it was abolished during perfusion of the blocker, 2 μM mecamylamine (middle trace), and the effect was reversible (right trace). In (b) inhibition of AChE with eserine (100 μM) caused a reversible increase in membrane potential noise recorded in a type 1 cell, located at the edge of a cluster (compare middle with left and right traces); the increase in membrane noise was due to the action of released ACh on nAChR, since it was abolished when 2 μM mecamylamine was present with eserine [compare middle traces from the same cell in (b) and (c)]. Thus, endogenous ACh release from type 1 cells may act on nicotinic autoreceptors to increase ACh noise when the esterase is inhibited.

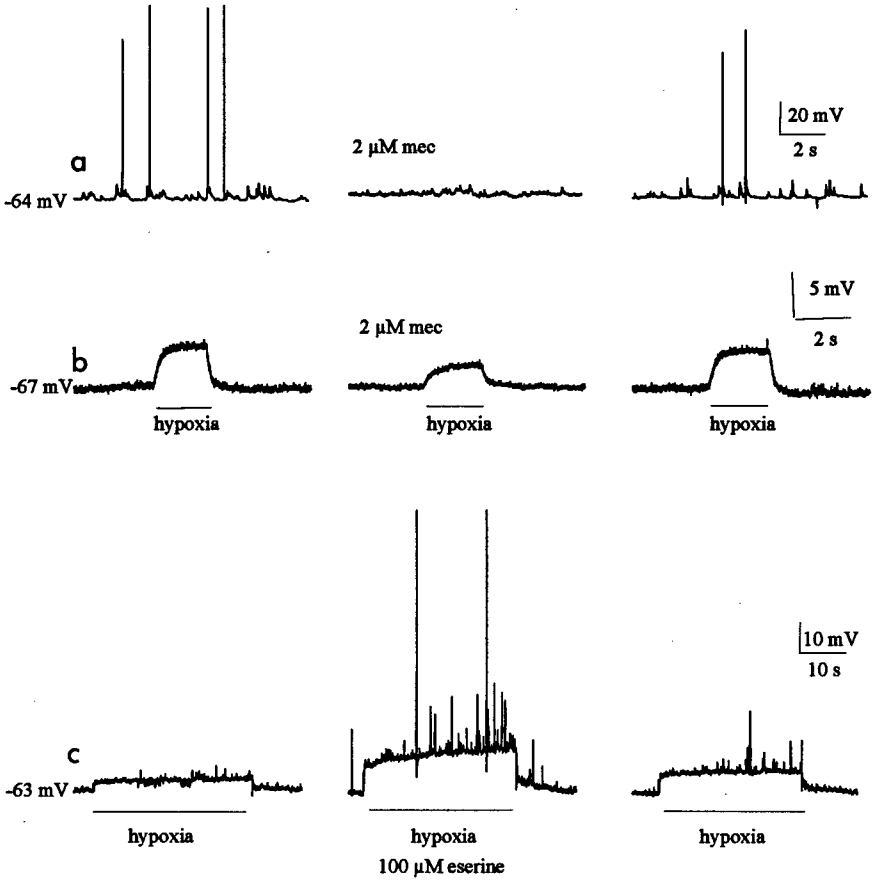

Fig. 4. Evidence for spontaneous and hypoxia-evoked ACh release from type 1 cells onto co-cultured PNs. The traces represent perforated-patch recordings of membrane potential from three different PNs (a, b, c), that were juxtaposed to type 1 cell clusters. In a, the spontaneous activity, comprising spikes and subthreshold potentials, was reversibly inhibited during perfusion of the nicotinic receptor blocker, 2 μM mecamylamine. In (b) hypoxia ($P_{O_2} = 5$ mmHg) caused membrane depolarization that was suppressed ($\approx 50\%$) by 2 μM mecamylamine (middle trace), and the effect was reversible following wash-out of the drug (right trace). In (c) the hypoxia-induced depolarization was potentiated (with resulting spike activity) in the presence of the AChE inhibitor, eserine (100 μM; middle trace), and the effect was reversible (right trace).

et al., 1997), suggests that ACh release is involved in chemical transmission between type 1 cells and co-cultured PNs.

3.4. Do petrosal neurons express receptors for ACh and other putative transmitters released by type 1 cells?

An additional requirement for ACh, and other putative type 1 cell mediators, to act as sensory transmitters in carotid body function is that their receptors must reside on petrosal afferent terminals. The authors have not yet addressed this question in a direct way, but have screened several potential excitatory transmitters, including ACh, for their effects on the cell bodies of isolated PNs. In a previous study it was found that $\approx 68\%$ of PNs expressed nAChR that were activated by nicotine or ACh applied from a 'puffer' pipette, and the response was markedly reduced or abolished by 100 μM hexamethonium (Zhong and Nurse, 1997a). As shown in Fig. 5a, the ACh-induced depolarization in responsive neurons is also inhibited ($> 90\%$) by the ganglionic blocker,

mecamylamine (2 μM). Thus, both PNs and type 1 cells express nAChR which share a similar pharmacology (Fig. 3a); however, it remains to be determined whether the nAChR on the two cell types are identical, i.e. share similar α and β subunits at the molecular level.

During recordings from 'juxtaposed' PNs in co-culture neither high concentrations of hexamethonium (100–200 μM; Zhong et al., 1997), nor 2 μM mecamylamine (Fig. 4a,b), blocked completely spontaneous synaptic activity and the hypoxia-evoked response. Since these drug concentrations almost completely eliminated the effects of puffer-applied ACh on the neuron (Fig. 5a; see also Zhong and Nurse, 1997a], it is likely that other transmitters contributed to the spontaneous and evoked responses. As a first step towards identifying these putative co-transmitters, the sensitivity of isolated PNs to other type 1 cell transmitter candidates was tested. So far, 5-HT and ATP produced rapid excitatory responses in many neurons (cultured without type 1 cells), and several were excited by all three transmitters, i.e. ACh, ATP and 5-HT. An example of one such neuron is shown in Fig. 5b. Moreover, it was recently shown that ≈43% of PNs were excited by 5-HT acting on MDL 72222-sensitive 5-HT_3 receptors; a minority population (≈6%) of neurons expressed 5-HT_2 receptors (Zhong et al., 1999). Thus it is plausible that co-release of ACh with ATP and/or 5-HT (or possibly other transmitters, e.g. substance P) could mediate hypoxic chemotransmission in the rat carotid body. These ideas are currently being tested in similar co-cultures.

4. Discussion

In this communication the authors review and extend the evidence for ACh as a major carotid body excitatory co-transmitter, released from type 1 receptor cells, during chemosensory signalling. In reviewing this evidence, it is instructive to ask

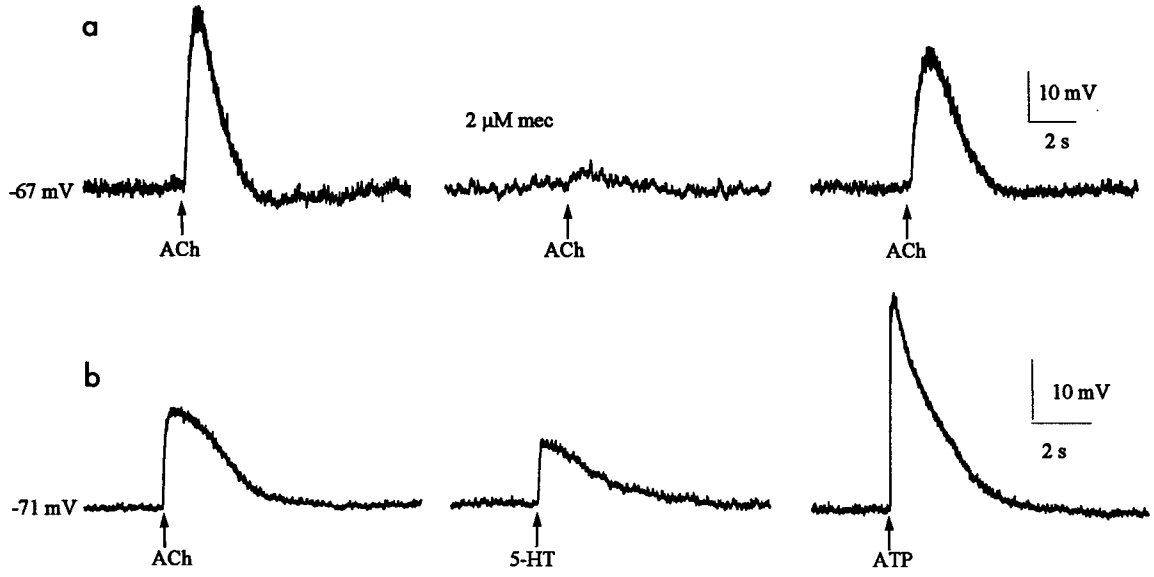

Fig. 5. Evidence that isolated PNs express receptors for ACh and other putative carotid body transmitters. In all cases (a, b), the transmitter was applied by pressure ejection from a 'puffer' pipette and the membrane potential was monitored by the perforated-patch technique. In (a) ACh caused membrane depolarization via activation of nicotinic AChR since the response was reversibly inhibited (>90%) in the presence of the blocker, 2 μM mecamylamine (middle trace); note mecamylamine inhibited the ACh response more strongly than the hypoxia-evoked response (Fig. 4b). In (b) the same neuron was excited by puffer-applied ACh (left), 5-HT (middle), and ATP (right), suggesting that several putative transmitters in type 1 cells could contribute to the chemosensory response. Pipette concentration of the applied transmitters was 1 mM in all cases.

at the outset whether ACh meets the requirements for a 'classical' transmitter in the carotid body, analogous to its established role at the skeletal neuromuscular junction (Katz, 1969). A first requirement, that type 1 cells possess the ability to synthesize, store, and degrade ACh appears to have been met, based on both in situ and in vitro studies. For example, Wang et al. (1989, 1992) used immunocytochemical techniques to show that the biosynthetic enzyme ChAT co-localizes with known type 1 cell markers in situ. In the present study, it is further shown that the vesicular transporter VAChT, which shares a common gene locus with ChAT and functions to accumulate ACh in synaptic vesicles (Eiden, 1998), is highly expressed in isolated rat type 1 cells in vitro (Fig. 2a). Clear cored vesicles (≈ 50 nm diameter), the storage sites for ACh, have been observed by electron microscopy near presynaptic dense projections in type 1 cells in situ (McDonald, 1981), as well as in the carotid body cultures (Fig. 1b,c; see also Nurse, 1987; Nurse et al., 1993). In addition, co-localization of the degradative enzyme, AChE to amine-containing type 1 cells has been demonstrated both in situ (Korkala and Waris, 1977; González et al., 1994) and in vitro (Nurse, 1987; see also Fig. 1a) by histochemical methods. The AChE reaction product was localized in the electron microscope to intracellular sites within type 1 cells, as well as to extracellular sites, at the boundaries of contiguous type 1 cells (Nurse, 1987). Together, these data provide strong biochemical and morphologic evidence for cholinergic functions in type 1 cells.

What is the physiological evidence that ACh is an excitatory sensory transmitter during carotid body excitation? Here, the evidence is controversial and there have been inconsistent reports even in preparations from the same species. For example, Eyzaguirre and Zapata (1968) found that stimulation of an upstream isolated cat carotid body preparation, increased the sensory discharge in a downstream (detector) preparation, and the effect was enhanced by the AChE inhibitor, eserine, and depressed by the nicotinic ganglionic blockers, hexamethonium or mecamylamine. More recently, a similar depressant action of mecamylamine on the hypoxic response of the perfused cat carotid body has been observed by Fitzgerald et al. (1995, 1997). In contrast, none of these treatments had any effect on chemosensory responses in earlier studies on the cat carotid body in vivo (Sampson, 1971; McQueen, 1977). It has also been suggested that attenuation of the hypoxic response by cholinergic blockers may be secondary to an attenuation of the carotid body response to CO_2 (Prabhakar, 1994).

While reasons for the above discrepancies remain uncertain (Fitzgerald et al., 1997), the authors obtained evidence for spontaneous and hypoxia-evoked ACh release in co-cultures of rat type 1 cells and PNs (see Zhong et al., 1997). Functional contacts developed in these co-cultures, and most importantly, subthreshold synaptic events were monitored in a significant proportion of PNs that were juxtaposed to type 1 clusters. Consistent with cholinergic transmission, the nicotinic blocker, hexamethonium (100–200 μM), partially inhibited spontaneous and hypoxia-evoked 'synaptic' responses in the neuron. As described in the present study, the authors have extended these findings and found that, not only was mecamylamine (2 μM) also effective in inhibiting these responses, but the hypoxia-evoked response was potentiated when AChE activity was inhibited with 100 μM eserine. Given the presence of extracellular AChE around type 1 clusters (Nurse, 1987; see also Fig. 1a), this is the expected result if type 1 cells released ACh at functional synapses with PNs, many of which are known to express nAChR (Zhong and Nurse, 1997a; see also Fig. 5a). Moreover, the authors obtained evidence for presynaptic functions of ACh since, in perforated-patch recordings from a few type 1 cells in cultures devoid of neurons, perfusion with eserine increased membrane potential noise, and the effect was blocked by mecamylamine. Taken together, these results suggest that both spontaneous and hypoxia-evoked ACh release can occur in these co-cultures, and ACh may act postsynaptically on nAChR present on PNs, and/or presynaptically on nicotinic ACh autoreceptors present on type 1 cells.

Despite the evidence presented here for a role of ACh in carotid body chemotransmission, its action appears to be that of a co-transmitter.

Notably, doses of ganglionic blockers (100–200 μM hexamethonium and 2 μM mecamylamine) expected to inhibit >90% of ACh-evoked responses in the petrosal soma (Zhong and Nurse, 1997a), only partially inhibited the hypoxia-evoked response (≈50% for 2 μM mecamylamine), and spontaneous subthreshold activity was still detectable (Zhong et al., 1997; see also Figs. 4a,b, 5a). These findings led us to investigate whether other putative type 1 cell transmitters might excite PNs. Indeed, 5-HT and ATP (which is often co-released with ACh and monoamines) excitated many PNs and, not infrequently, the same neuron may be excited by all three agents, ACh, 5-HT and ATP (Fig. 5b; authors unpublished observations). The current goal is to test whether the spontaneous events and the hypoxia-evoked response in co-cultured PNs can be completely inhibited by different combinations of the corresponding receptor blockers. Ultimately, a satisfactory resolution to the 'transmitter problem' will require that the same blockers be demonstrated to be effective in the intact carotid body -sinus nerve preparation. However, it is also possible that other neuroactive agents released from type 1 cells during chemosensory stimulation may modulate the firing frequency of CSN fibers via regulation of voltage-dependent ionic currents, e.g. hyperpolarization-activated cation current, I_h (Stea and Nurse, 1992; Zhong and Nurse, 1997b).

In summary, it might be useful to reflect on the rather slow progress made towards understanding carotid body chemotransmission since the 1930s. On reviewing the evidence in 1954, W.W. Douglas likened the arguments in favour of cholinergic transmission in the carotid body to "an old-fashioned stool upon three legs". He concluded that: "With one leg hollow, one wobbly and one missing, the argument that ACh is involved in chemosensory transmission appears to me to stand but poorly at present; but the theory is an attractive one and the authors have no convincing evidence against it". Though much remains to be resolved, use of perfused and isolated carotid body–nerve preparations in the intervening years, together with the more recent introduction of the co-culture technique described here, have undoubtedly helped to put the 'cholinergic hypothesis' on firmer ground.

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RESPIRATION PHYSIOLOGY

Neuroepithelial bodies as airway oxygen sensors

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Abstract

Since the discovery of neuroepithelial bodies (NEB) in the late 1930s, evidence has accumulated to suggest that these cells may function as hypoxia-sensitive airway sensors. Until recently, this hypothesis was based largely on morphological observations. The use of in vitro models of isolated NEB, combined with electrophysiological approaches, have provided direct evidence that NEB cells express a membrane-bound O_2 sensor and are the transducers of hypoxic stimulus. Here, we review the historical evidence and current state of knowledge of the oxygen-sensing properties of NEB cells, comparison with other O_2 sensing cells, as well as recent advances that have been made using molecular and electrophysiological techniques. The possible role of NEB in perinatal pulmonary pathophysiology is also discussed.

Keywords: Neuroepithelial bodies; Airway chemoreceptors; NADPH oxidase; O_2-sensitive K^+ current; Pediatric pulmonary disorders

1. Introduction

Over 50 years ago, the existence of solitary and grouped cells with a clear cytoplasm and 'neuroendocrine' characteristics, widely dispersed throughout the airway epithelium of mammals, were discovered in mammalian lungs. In the early seventies, Lauweryns and Peuskens designated innervated clusters of these cells as intrapulmonary neuroepithelial bodies (NEB) (Lauweryns and Cokelaere, 1973). These innervated epithelial corpuscles have since been characterized in the lungs of humans, various mammals and amphibians (Cutz et al., 1984, 1995; Sorokin and Hoyt, 1989; Adriaensen and Scheuermann, 1993). In a series of experiments using rabbit neonates, Lauweryns and colleagues have shown that NEB react to airway hypoxia by increased exocytosis of its dense core vesicles and decrease in cytoplasmic amine content, suggesting that these cells may

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represent hypoxia-sensitive airway sensors (Lauweryns and Cokelaere, 1973; Lauweryns et al., 1977, 1978). It has been estimated that NEB represent < 1% of epithelial cells in human lungs (Cutz et al., 1984). This relatively small number of NEB and widespread distribution in a complex organ with difficult access has slowed our progress in unraveling their precise function. Given these obstacles, the general consensus is that NEB may function as hypoxia-sensitive airway sensors, and together with the principal arterial chemoreceptor (i.e. the carotid body), initiate sensory inputs to affect the control of respiration.

Sorokin and Hoyt (1989, 1990) have previously discussed some of the supposed functions of NEB in mammalian lungs including: (1) the ability of NEB to function as transducers, i.e. they translate the airway 'chemical' environment (e.g. hypoxia) into a change in electric current by releasing mediators at the basal pole; (2) modulation of bronchomotor tone via targeting bronchial smooth muscle and the associated nerves located directly beneath NEB; (3) promotion and regulation of the growth of developing airways by stimulating the proliferation of local endoderm; (4) release of amine and peptide modulators in response to increasing fetal hypoxia near term in order to maintain vasoconstriction in the pulmonary circuit; and (5) neonatal respiratory adaptation. Here we briefly overview the structural and functional data as well as recent cellular and molecular studies on O_2-sensing properties of NEB cells. The possible involvement of NEB in the pathophysiology of some neonatal/pediatric pulmonary disorders is also discussed.

2. Morphological and experimental evidence

Morphologic and experimental studies to support NEB function as hypoxia-sensitive airway chemoreceptors modulated by the central nervous system include: (a) preferential location of NEB at airway branching points (Fig. 1); (b) apical microvilli in contact with the airway lumen; (c) cytoplasmic neurosecretory granules (Fig. 2) containing monoamine and neuropeptides (e.g. gastrin-releasing peptide (GRP), bombesin, calcitonin, calcitonin gene-related peptide (CGRP), CCK, endothelin, and amine (serotonin, 5-HT)); (d) afferent sensory innervation derived from the vagus nerve; and (e) proximity to blood capillaries (Lauweryns and Cokelaere, 1973; Lauweryns et al., 1977, 1985; Sorokin and Hoyt, 1989; Cutz et al., 1995).

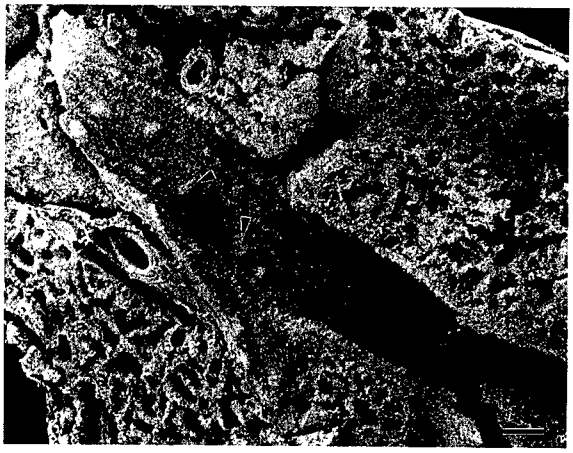

Fig. 1. Scanning electron micrograph of the central airway of a hamster fetal lung (15 day gestation) with numerous NEB structures (arrow heads) protruding into the airway lumen, particularly at airway branch points. Scale bar > 100 μm.

The early experimental studies demonstrated that exposure of neonatal rabbits to short-term

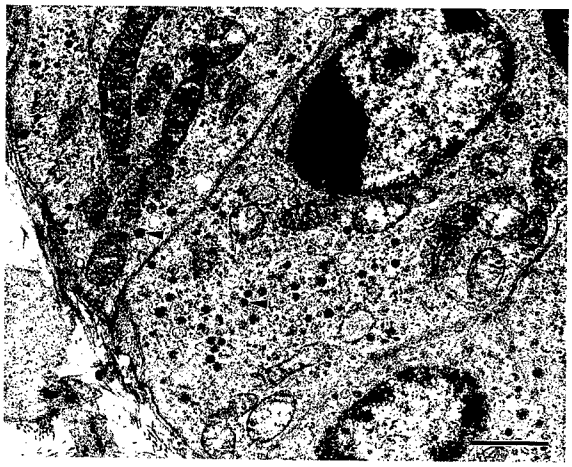

Fig. 2. Transmission electron micrograph of a NEB cell in hamster fetal lung (15 day gestation). The cytoplasm contains numerous dense-core vesicles (arrow heads) concentrated near the basement membrane. Scale bar > 0.5 μm.

hypoxia (10% O_2 in N_2 for 20 min), but not hyperoxia, resulted in increased exocytosis of neurosecretory granules and decreased amine fluorescence in NEB cells, implying hypoxia-evoked amine (serotonin) secretion (Lauweryns and Cokelaere, 1973). Subsequent cross-circulation studies confirmed that this secretory response was due to airway hypoxia rather than by hypoxaemia of the pulmonary blood (Lauweryns et al., 1978). This hypoxia-evoked release of 5-HT and neuropeptides may produce, however, a local vasoconstriction in hypoxically aerated lung areas, shunting blood from poor to better ventilated parts of the lung.

The micro-anatomy of NEB innervation and its effects on modulation of the hypoxic response was investigated using various vagotomy and vagal stimulation procedures (Lauweryns et al., 1985, 1987). Long-term infranodosal, but not supranodosal, vagotomy resulted in degeneration of most intracorpuscular nerve endings in rabbit NEB, indicating that NEB are predominantly innervated by sensory nerve fibers, derived from cell bodies in the nodose ganglion of the vagus nerve (Lauweryns et al., 1985). When the vagus nerve was cut and electrically stimulated for 10 min there was an amplitude-dependent increase in serotonin content of NEB and decrease in the number of exocytotic dense-core vesicle (DCV) profiles, effects opposite to those seen after exposing NEB to hypoxia (Lauweryns et al., 1987). In addition, acute hypoxia caused a depletion of synaptic vesicles and an increase in the amount of membrane-bound cisternae and multivesicular bodies in morphologically efferent nerve endings (Lauweryns and Van Lommel, 1982). The latter whole animal experiments suggested that NEB cell function may be modulated by the CNS via their efferent-like nerve endings. In addition, the secretory response of NEB to acute hypoxia was modified after long-term (3 days) infranodose vagotomy (Lauweryns and Van Lommel, 1986). The cytoplasmic fluorescence (i.e. amine content) increased and basal exocytosis remained unchanged, suggesting that the hypoxic NEB secretory response was neurally controlled possibly by intrapulmonary axon reflexes in sensory nerve fibers (Lauweryns and Van Lommel, 1986; Adriaensen and Scheuermann, 1993).

Recently, nerve tracing experiments, using the fluorescent anterograde neural tracer DiI and confocal laser scanning microscopy, support the previous morphological findings favouring an extensive vagal sensory innervation of NEB (Adriaensen et al., 1997). Following injection of DiI into the nodose ganglion of adult rats, extensive intraepithelial terminal arborizations of DiI-labeled vagal afferents were co-localized with CGRP-immunoreactive NEBs, visualized using immunocytochemistry and confocal microscopy on thick frozen sections. Also, serial ultrastructural sections of rabbit NEB have shown that based on morphological criteria the efferent and afferent nerve endings are often, but not always, part of the same nerve process (Lauweryns et al., 1985). In summary, these studies suggest that innervation of NEB is mainly sensory but might permit modulation of neuroepithelial responses by local axon reflexes.

Further morphological support for the role of NEB as hypoxia-sensitive airway sensors stems from studies on the effects of chronic hypoxia, since it is well documented that exposure of animals to chronic hypoxia evokes cellular hyperplasia and hypertrophy in peripheral chemoreceptors (McDonald, 1981; Dhillon et al., 1984; McGregor et al., 1988). Adult Sprague Dawley rats (but not Wistar rats) exposed to chronic normobaric hypoxia showed a significant increase in the number of solitary pulmonary neuroendocrine cells (PNEC) and enlargement of NEB to more than double that of control rats (Pack et al., 1986). On the other hand, Wistar rats maintained in hypoxia between one to three weeks showed elevated levels of intracellular CGRP without a change in NEB cell numbers. An increased number of NEB has also been reported in young rabbits kept in hypobaric chambers or raised at high altitude. While there are some contradictory findings, the overall consensus is that NEB cells are activated by and become adapted to this natural stimulus, chronic hypoxia (reviewed in Cutz, 1997).

With the establishment of a reproducible in vitro system to isolate NEB cells (Cutz et al., 1993), it became possible to further test their function as airway sensors in a controlled oxygen

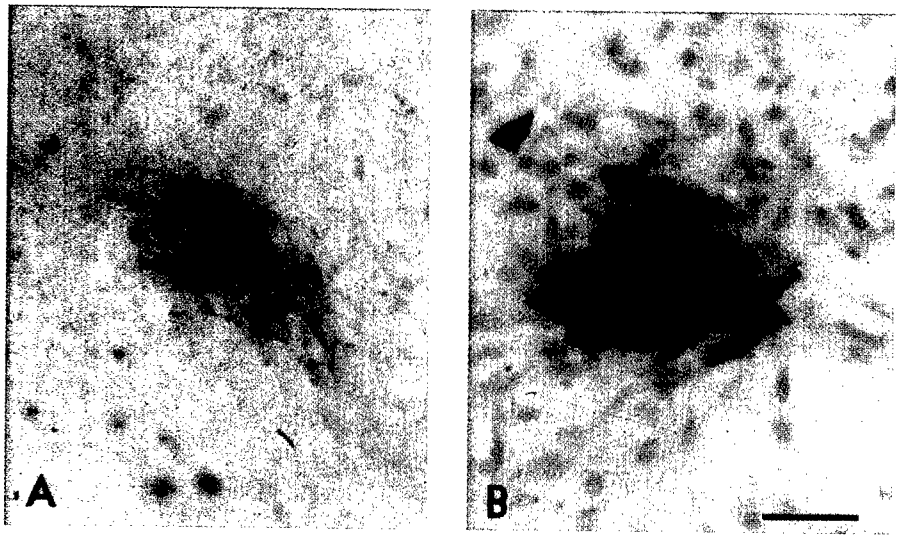

Fig. 3. NEB from dissociated rabbit fetal lung (26 day gestation) after 3 days in culture. (A) Vital staining with neutral red shows positive staining (red cytoplasm with nucleus remaining unstained) in NEB cells. (B) Paraformaldehyde-fixed NEB showing positive immunostaining for serotonin. Scale bar > 50 μm.

environment. Exposure of NEB cell cultures from fetal rabbit lung to moderate hypoxia ($Po_2 > 50$ torr) resulted in a 15-20% reduction in intracellular 5-HT content after 5 to 15 min, whereas severe hypoxia ($Po_2 > 20$ torr) caused a reduction up to 80% (Cutz et al., 1993). This decrease in intracellular content of 5-HT was measured with HPLC and electrochemical detection. Given a 60 min recovery period following exposure to severe hypoxia, the 5-HT level returned to control levels, suggesting modulation of 5-HT content under ambient O_2 concentration. As predicted, ultrastructural analysis of hypoxia exposed cultures confirmed increased exocytosis of dense-core vesicles (DCV) from NEB cells compared with controls (Cutz et al., 1993). These in vitro studies confirmed the secretory response of NEB cells to hypoxia seen previously in vivo, but in the absence of innervation. In addition, exposure of normoxic NEB cultures to Ca^{2+} ionophore (A23187), which increases intracellular Ca^{2+}, also resulted in the reduction of 5-HT content as well as increased exocytosis of DCV, showing that NEB require an increase in Ca^{2+} for stimulus-secretion coupling (Cutz et al., 1993). Further biochemical, physiological and molecular details of O_2-sensing by NEB cells have been realized using this in vitro model system (see below).

3. Characterization of O_2 sensing mechanism in neuroepithelial body cells

3.1. Ionic currents

Until recently research assessing the O_2-sensing capabilities of NEBs has been limited to morphometric and ultrastructural studies, e.g. measuring changes in dense-core granule content. With the development of an in vitro model of isolated NEB cells (Cutz et al., 1993), and by devising a means to identify NEB in living state using neutral red, we began to investigate their membrane physiology and responses to different oxygen environments (Fig. 3). Using the whole-cell patch clamp technique we have shown that NEB cells isolated from fetal rabbit lungs exhibit membrane properties of excitable cells (Youngson et al., 1993). Depolarizing voltage steps from a holding potential of -60 to $+20$ mV activated both a fast transient inward current and a prolonged outward current (Fig. 4A,B). Characterization of these currents revealed that fetal NEB cells express voltage-activated K^+, Na^+, and Ca^{2+} currents

(Youngson et al., 1993). Upon exposure to hypoxia (Po_2 25–30 mmHg) there was a reversible reduction (25–30%) in outward K^+ current, with no change in inward Na^+ and Ca^{2+} currents (Fig. 4C,D). In current clamp, closure of K^+ channels by hypoxia resulted in an increase in the spontaneous firing frequency and slope of the depolarizing pacemaker potential in NEB cells.

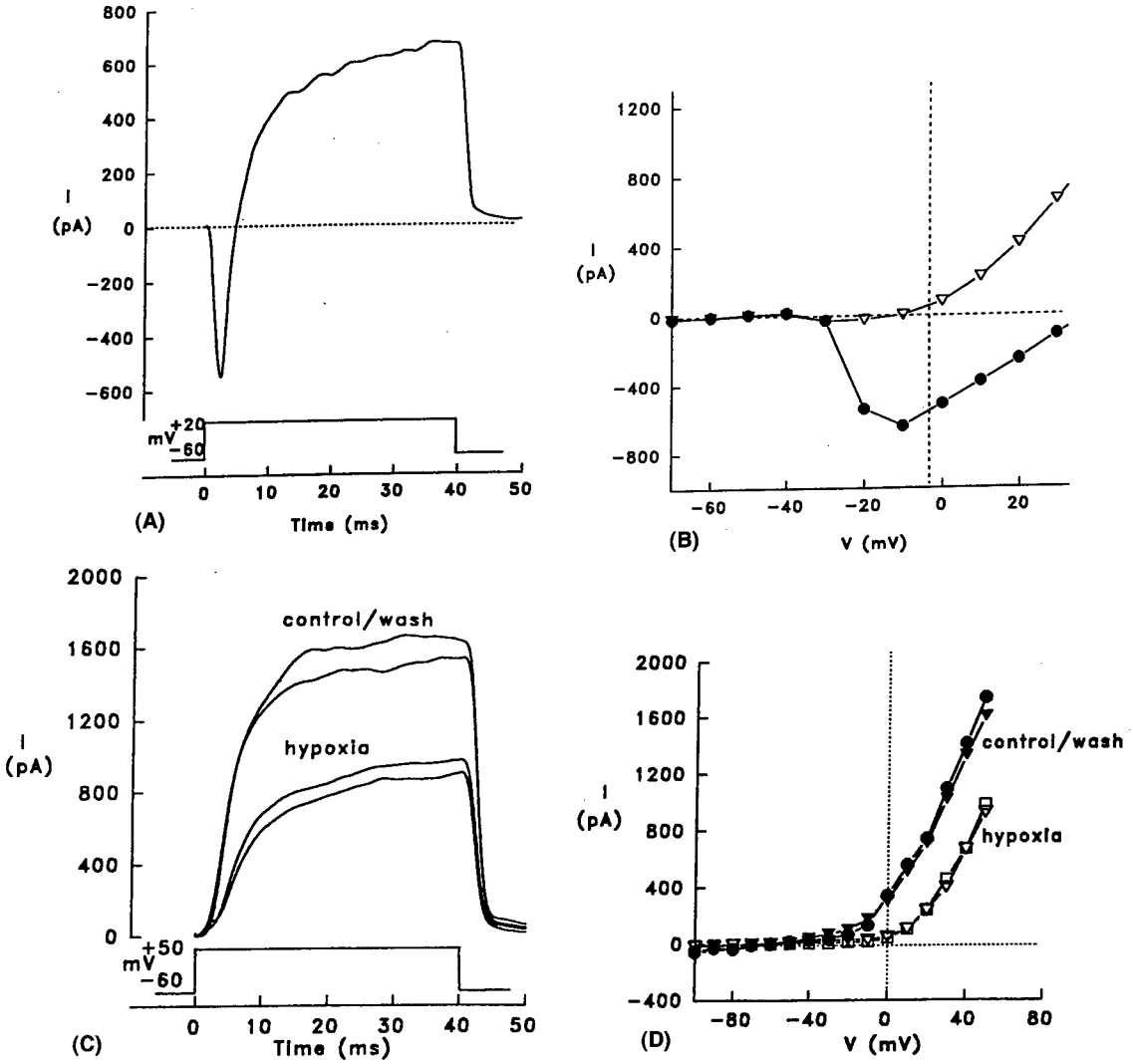

Fig. 4. (A) Whole-cell voltage-activated currents recorded from rabbit fetal NEB cell culture (as in Fig. 3) under voltage clamp. With a holding potential of −60 mV and a pulse duration of 40 msec, a voltage step to +20 mV showed the presence of both an inward and outward current. Although the inward current was transient, the outward current lasted for the entire duration of the 40 msec pulse. (B) I–V relation for a cultured NEB cell. Voltage clamp currents were measured from a holding potential of −60 mV to various test potentials. The outward (open triangles) and inward (closed circles) currents were activated between −20 and −30 mV and peaked at approximately −10 mV. (C) Effect of hypoxia on outward K^+ current in NEB cell. K^+ current was recorded during voltage step to +50 mV from a holding potential of −60 mV. When NEB cells were exposed to hypoxic bathing medium (Po_2; 25–30 mmHg) there was a 25–30% reduction in the outward K^+ current. This was recoverable when cells were returned to normoxic conditions (Po_2; 150 mmHg) followed by an additional reduction of K^+ current with similar magnitude to the first with hypoxic exposure. (D) Current voltage relationship of cell in C. [Panels A, C and D were reproduced with permission from *Nature* (Youngson et al., 1993). Panel B was reproduced with permission from *Landes Bioscience* (Youngson et al., 1997b)].

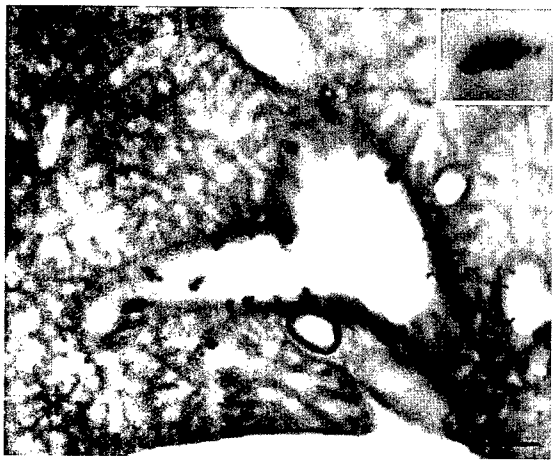

Fig. 5. Serotonin-immunoreactive NEB in a 300–400 μm thick vibrotome section from a hamster fetal lung (15 day gestation). This fresh lung section was fixed with 4% paraformaldehyde and stained for 5-HT, using the immunoperoxidase method, in order to illustrate the distribution of NEB within the airway. Scale bar > 100 μm. Insert: Higher magnification of NEB with tall columnar cells extending into airway lumen.

The finding that a component of the K^+ current is sensitive to hypoxia suggests that NEB cells might function as transducers of airway hypoxia. Similar O_2-sensitive K^+ currents have been well characterized in carotid body glomus cells (López-Barneo et al., 1988; Ganfornina and López-Barneo, 1992).

Recently, a neonatal rabbit lung slice preparation has been developed for further characterization of voltage-dependent ionic currents and hypoxic chemosensitivity of 'intact' NEBs in situ (Fu et al., 1999). Similar to NEB cells grown in dispersed fetal lung culture (Youngson et al., 1993), intact neonatal NEBs in fresh lung slices (200–400 μm thick) (Fig. 5) responded to hypoxia (Po_2 15–20 mmHg) with a reduction (34%) in outward K^+ current, during voltage steps from −60 to +30 mV. Inward currents, carried by Na^+ and Ca^{2+} ions, also failed to respond to hypoxia in lung slice preparations. In addition, neonatal NEBs in situ demonstrated both Ca^{2+}-dependent ($I_{K(Ca)}$) and Ca^{2+}-insensitive, voltage-dependent ($I_{K(V)}$) K^+ currents, both of which were equally suppressed by hypoxia. No further inhibition of K^+ currents was seen with either TEA and 4-AP plus hypoxia, suggesting that TEA/4-AP-sensitive K^+ current may correspond to O_2-sensitive K^+ current in rabbit neonatal NEB cells. Hence, this new lung slice preparation is a promising advance in the study of electrophysiological properties of NEB cells, providing access to intact NEBs that are scattered throughout the airway epithelium.

Recently, O_2-sensitive K^+ channels have been characterized in H-146 small cell lung carcinoma (SCLC) cells (O'Kelly et al., 1998). SCLC cells are believed to be derived from the same population of committed pulmonary neuroendocrine precursor cells that give rise to NEB cells. Using whole-cell patch clamp, voltage-gated K^+, Na^+, and Ca^{2+} currents were identified in H-146 cells and a Ca^{2+}-insensitive component of the K^+ current was reversibly inhibited by hypoxia. This O_2-sensitive K^+ channel contributed to the resting membrane potential and its inhibition gave rise to cell depolarization.

3.2. O_2 sensor protein

Cross et al. (1990) and Acker and Xue (1995) postulated that oxygen chemotransduction in the carotid body glomus cell is initiated at a membrane bound O_2 sensor, namely the haem-linked nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, similar to the one identified in professional phagocytes (neutrophils, macrophages, eosinophils and monocytes) (Cross and Jones, 1991). Upon stimulation, phagocytes assemble a multi-subunit NADPH oxidase which is able to catalyze the one-electron reduction of molecular oxygen to superoxide (O_2^-), using electrons supplied by NADPH (Cross and Jones, 1991). Superoxide and its oxidant derivatives (e.g. hydrogen peroxide) are then used to kill bacteria and fungi invading the host. The oxidase components include two membrane proteins ($gp91^{phox}$ and $p22^{phox}$) that together form a b-type cytochrome and two cytosolic peptides ($p47^{phox}$ and $p67^{phox}$) (Cross and Jones, 1991). Rac 2, a cytosolic small GTPase, also appears to be required for oxidase activity. Using immunocytochemistry, most of the NADPH oxidase components were shown to be expressed in NEB cells of fetal rabbit lung as well as in glomus cells of rat and human

carotid bodies (Youngson et al., 1993, 1997a; Kummer and Acker, 1995). NADPH oxidase in rabbit NEB cells exhibited activity under basal conditions, as shown using microfluorometry with dihydrorhodamine 123 used as a probe to assess H_2O_2 generation (Wang et al., 1996). Production of H_2O_2 was significantly stimulated by exposure of NEB cells to phorbol ester and inhibited by diphenylene iodonium (DPI). Phorbol ester stimulated phosphorylation (i.e. activation) of the cytosolic components of the oxidase, while DPI inhibits oxidase function through inhibition of flavoprotein activity.

If the NADPH oxidase-O_2 sensitive K^+ channel complex functions as an O_2 sensor in NEB cells, then one might expect the gating properties of O_2-sensitive K^+ channels to be governed by the byproducts of oxidase activity, e.g. H_2O_2. Indeed, external application of H_2O_2 on NEB cells in culture or in fresh lung slices under normoxic conditions resulted in an increased outward K^+ current, indicating that the KH_2O_2 channel expressed on NEB cell membrane can be regulated by reactive oxygen intermediates produced by the oxidase (Wang et al., 1996; Fu et al., 1999). Using whole-cell voltage clamp it was shown that the K^+ current of cultured fetal rabbit NEB cells exhibited inactivating properties similar to KV3.3a transcripts expressed in Xenopus ooctye model system (Vega-Saenz de Miera and Rudy, 1992). Kv3.3 is a H_2O_2-sensitive K^+ channel that belongs to the Shaw family of K^+ channels and initial studies have localized its transcripts to brain, lung and kidney (Vega-Saenz de Miera et al., 1994). Importantly, mRNAs for both the H_2O_2-sensitive, voltage-gated K^+ channel subunit (KH_2O_2) KV3.3a and membrane components ($gp91^{phox}$ and $p22^{phox}$) of NADPH oxidase are coexpressed in NEB cells of fetal rabbit and neonatal human lungs.

In theory, inhibition of oxidase activity by DPI should mimic hypoxia, resulting in suppression of outward K^+ current, and application of DPI during acute hypoxia should produce the same degree of K^+ current suppression as measured with DPI or hypoxia alone. In both dispersed lung cultures from fetal rabbit (Youngson et al., 1993) and fresh lung slices from neonatal rabbits (Fu et al., 1999), DPI at low concentration (1 μM) suppressed K^+ current in NEB cells, similar in magnitude to that seen with hypoxia; furthermore, after treatment with DPI, NEB cells no longer responded to hypoxia. Similarly, in isolated perfused carotid body preparations, DPI transiently increased spontaneous discharge and blocked hypoxia-induced increases in neural discharge (Cross et al., 1990). While these effects of DPI on the intact carotid body support the involvement of H_2O_2 in hypoxic chemotransduction, this drug has been shown to be a non-selective blocker of K^+ and Ca^{2+} currents when used at a higher concentration (3–10 μM) in isolated rat glomus cells (Wyatt et al., 1994) or rat pulmonary artery myocytes (Weir et al., 1994). Hence, DPI can mimic the actions of hypoxia by acting at numerous sites along the transduction pathway in O_2 chemoreceptors. These findings, together with the demonstration that low pO_2 decreases the open probability of K^+ channels in excised membrane patches from glomus cells (Ganfornina and López-Barneo, 1991), suggest a direct link between the O_2 sensor protein complex (NADPH oxidase) and O_2-sensitive K^+ channels. The functional link between the oxidase and O_2-sensitive K^+ channel has been further strengthened by recent observations on NEBs in oxidase deficient (gp91 knock-out) mice. In this model, voltage-activated K^+ channels in NEB cells exhibit appropriate pharmacological and electrophysiological properties, including responses to external application of H_2O_2, but fail to respond to hypoxia or application of DPI (Fu et al., 1998).

3.3. Model of O_2 sensing in neuroepithelial body cells

Several models of O_2 sensing by chemoreceptor cells have been proposed (Remmers and Lahiri, 1998). The preferred mechanism of O_2 chemotransduction in NEB cells (Fig. 6) is based on one of the models proposed for carotid body glomus cells (López-Barneo et al., 1988; González et al., 1992; López-Barneo, 1996). In the 'membrane model' a primary event in response to a decrease in Po_2 is the closure of O_2-sensitive K^+ channels (López-Barneo et al., 1988; López-Barneo, 1996).

The actual O_2 sensor in glomus and NEB cells is thought to be a haem-linked NADPH oxidase which is closely associated with O_2-sensitive K^+ channel subunit (Acker et al., 1992; Acker and Xue, 1995; López-Barneo, 1996; Wang et al., 1996). During hypoxia, NADPH oxidase fails to maintain superoxide (and hence H_2O_2) production and as a result cannot keep redox couples preferentially in the oxidized state. Modulation of the redox status of a cysteine residue in the K^+ channel protein (Ruppersberg et al., 1991) may, in turn, alter the protein conformation of this H_2O_2-sensitive K^+ channel, leading to channel closure, and a decrease in the outward flow of K^+ ions. The resulting membrane depolarization leads to activation of voltage-sensitive Ca^{2+} channels, followed by an influx of extracellular Ca^{2+}. The increase in intracellular Ca^{2+} triggers a sequence of events leading to the secretion of neurotransmitter(s) onto terminals of sensory nerve fibers in contact with NEB cells which, in turn, relay afferent impulses to the brain stem.

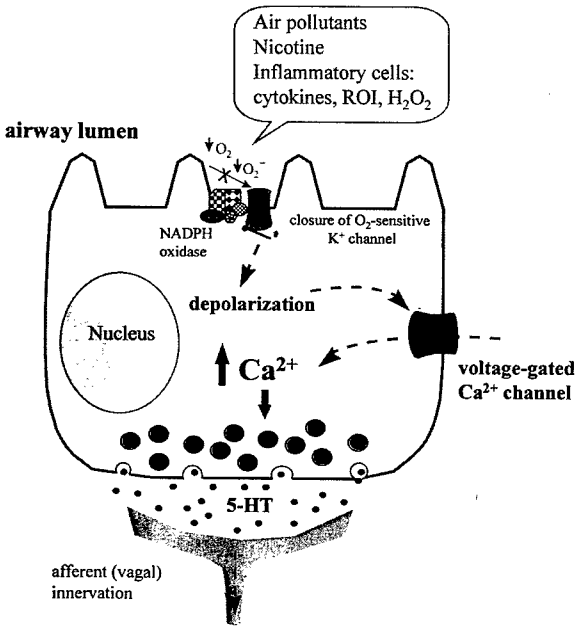

Fig. 6. Proposed mechanism of chemotransduction in NEB cells. The reduced availability of O_2 substrate with hypoxia causes a reduction in the level of byproducts of O_2 reduction by NADPH oxidase (i.e. O_2^- and H_2O_2). This, in turn, alters the redox potential of the cells, causing a change in the protein conformation of the O_2-sensitive K^+ channel. The result is a closure of these channels preventing the outward flux of K^+ ions, leading to membrane depolarization, calcium influx and neurotransmitter secretion. The binding of neurotransmitter to sensory afferent terminals of the vagus nerve causes a relay of information to the respiratory centers in the brain stem. Direct contact with the airway exposes NEB cells to air pollutants, a multitude of chemicals in cigarette smoke, including nicotine, and inflammatory cytokines and reactive oxygen intermediates (e.g. H_2O_2). All of these 'exogenous stressors' could directly or indirectly alter O_2 sensor function in NEB cells. The failure to signal airway hypoxia could in turn initiate a chain of events leading to SIDS.

4. Comparison of neuroepithelial bodies with other O_2 sensing cells

NEB are highly organized clusters of specialized cells with 'neuroendocrine' characteristics arranged into organoids that are widely dispersed throughout the epithelium of the bronchial tree. Their sensory innervation, amine and peptide content, proximity to blood capillaries, prevalence at bronchiolar bifurcations, and direct exposure to inspired gases, suggest that NEB may function as airway O_2 sensors. Similarly, the principal arterial chemoreceptors, carotid bodies, are strategically located at the bifurcation of the common carotid arteries. At this site, carotid body chemoreceptors monitor the Pa_{O2}, Pa_{CO2} and pH of blood en route to the brain. Carotid chemosensory cells, type 1 (or glomus) cells, are also arranged in clusters like NEB cells, but within a compact and structurally complex organ that is relatively small. Both glomus and NEB cells are in close, synaptic contact with the endings of afferent chemosensory fibers. However, only glomus cells are perfused at high rate with blood via a dense network of capillaries. Interestingly, in both rat carotid body (McDonald, 1981) and rabbit NEB (Lauweryns and Van Lommel, 1982), exposure to hypoxia induces synaptic activity in motor-like varicosities of the sensory nerve fiber.

The comparisons between NEBs and carotid bodies extend beyond the morphological level since they both respond to acute hypoxia via similar membrane bound and intracellular signaling pathways (see above, Table 1). Whereas glomus cells respond to both hypoxia and

Table 1
Comparison of morphological and physiological features of carotid body and pulmonary NEB

Features	Carotid bodies	Neuroepithelial bodies (NEB)
Embryonic origin	Neural crest (Pearse et al., 1973)	Foregut endoderm (Sorokin and Hoyt, 1997)
Location and distribution	Paired, compact organ near bifurcation of common carotid artery	Clusters/organoids, widely dispersed throughout the airway epithelium, particularly at airway bifurcations
Blood flow normalized to tissue weight	Extremely high	Moderate
Sensory cell population (cell number)	~8700 glomus cells/rat carotid body; determined by serial sectioning (McDonald, 1981)	~8000 NEB or 74 000 NEB cells/pair in adult hamster lungs (Hoyt et al., 1982)
Primary chemosensory unit	Clusters of glomus (type 1) cells innervated by afferent (sensory) fibers of the carotid sinus nerve	NEB cells innervated by afferent (sensory) fibers of vagus nerve
Contents of DCV*	Dopamine, norepinephrine, serotonin, acetylcholine, substance P, atrial natriuretic peptide, Leu-, Met-enkephalin (González et al., 1994)	Serotonin, CGRP, GRP, CCK, Leu-enkephalin, substance P, calcitonin (Cutz et al., 1995)
Secretion	Contents of dense core vesicles (DCVs) released onto closely apposed nerve terminals	Contents of DCVs released from basal pole of NEB cells onto apposed nerve terminals, bronchial smooth muscle cells and into pulmonary circulation
Function	Arterial chemoreceptor (sensitive to changes in blood Po_2, Pco_2, and pH)	Pulmonary airway oxygen sensor (?), no response to hypercapnia. Paracrine glands that affect local changes in pulmonary blood flow and regulate lung development
Membrane components involved in O_2-sensing* (see Peers, 1997; Fu et al., 1999)	*O_2-sensitive K^+ channel*: Hypoxia causes suppression of (a) lower conductance (40 pS), voltage-gated, Ca^{2+}-insensitive K^+ current (I_{Ko2}) in adult rabbit (b) high conductance (190 pS), charybdotoxin-sensitive, Ca^{2+}-activated K^+ current (I_{BKCa}) in neonatal rat (c) voltage and charybdotoxin-insensitive, low conductance 'leak' K^+ current in neonatal rat *O_2-sensing protein*: NADPH oxidase, present	*O_2-sensitive K^+ channel*: Hypoxia causes suppression of (a) Ca^{2+}-independent K^+ current ($I_{K(V)}$) (b) Ca^{2+}-dependent K^+ current ($I_{K(Ca)}$) DPI and H_2O_2 modulate outward K^+current *O_2-sensing protein*: NADPH oxidase, present
Hypoxia-induced membrane depolarization	Yes	Yes
Hypoxia-evoked increase in cytosolic calcium	Yes	Not available

* Species variability.

hypercapnia with neurotransmitter (e.g. dopamine) secretion (González et al., 1994), NEB cells respond to acute hypoxia, but apparently not to hypercapnia, with degranulation of DCV and release of 5-HT (Lauweryns et al., 1977, 1990; Cutz et al., 1993). Therefore, lung chemoreceptors may complement rather than duplicate carotid body activity, especially in the neonatal period.

The prominence of NEB during the perinatal period (Cutz et al., 1984), as well as the association of a variety of pediatric disorders, such as sudden infant death syndrome, bronchopulmonary dysplasia and apnea of prematurity, with changes in NEB cell number (see below), suggest that NEB cells function in the control of respiration during the transition from intrauterine environment to air breathing. At birth, there is a dramatic increase in Pa_{O2} and this relative hyperoxia silences carotid chemoreceptor function. Over the next several days, carotid chemoreceptors slowly develop a vigorous response to hypoxia, except that the range of O_2 sensitivity has shifted to the right (more sensitive) compared to the fetal response. It is widely accepted that the rise in O_2 tension at birth is the trigger for this shift of O_2 sensitivity or 'resetting' of arterial chemoreceptors (González et al., 1994). During infancy the strength of this ventilatory response to hypoxia continues to increase until the sensitivity of carotid chemoreceptors has reset to the high Pa_{O2} of the adult. Perhaps the function of airway O_2 sensors in the neonate provides protection against hypoxia while the sensitivity of carotid chemoreception is resetting towards adult values. It has been pointed out that NEB in the lung may provide an early warning system which may be particularly important in animal species whose lungs are relatively immature at birth and have a less efficient gas exchange mechanism. This is consistent with the finding of prominent NEB in neonatal rabbits, hamsters, other rodents, primates and carnivores (Cutz et al., 1995).

The second level of O_2 sensing in the lung involves the pulmonary vascular bed, particularly the peripheral 'resistance' arterial bed. While hypoxia causes dilatation of systemic arteries, it causes constriction in the small pulmonary arteries. Hypoxic pulmonary vasoconstriction (HPV) is a unique response which is essential for the control of pulmonary blood flow in the fetus and is the mechanism by which local lung perfusion is matched to ventilation in the adult to maintain systemic Po_2. Hypoxic constriction has been demonstrated in single pulmonary vascular smooth muscle cells (PVSMC) and, like carotid body glomus cells, hypoxia has been shown to inhibit outward K^+ current, thus causing membrane depolarization and calcium entry through voltage-dependent calcium channels (Weir and Archer, 1995). It is this increase in free cytosolic calcium that leads to activation of the contractile apparatus in PVSMC. In addition, PVSMC are equipped with an intrinsic O_2 sensor (a redox-based O_2 sensor) and treatment of these cells with DPI inhibits whole-cell K^+ current (Weir et al., 1994). However, DPI inhibits, rather than enhances, HPV and this response is likely due to its action as a nonspecific calcium channel blocker (Weir et al., 1994). Hence, there are striking similarities in the O_2-sensing mechanisms in PVSMC, glomus cells of the carotid body and NEB cells. Finally, it is important to keep in mind that potent vasoactive agents (e.g. 5-HT, bombesin, CGRP) secreted by NEB cells during hypoxia may modulate pulmonary blood flow by binding to receptors on PVSMC of pulmonary arteries.

5. The possible role of neuroepithelial bodies in neonatal/pediatric lung disease

The interest in the study of NEB in various pulmonary diseases was prompted in part by potential insight which could be gained from these 'experiments of nature'. Furthermore, unlike carotid body chemoreceptors which are enclosed and protected within the vascular system, NEB are in direct contact with external milieu (i.e. gases, air pollutants) as well as are subject to influences and interactions with other cells (e.g. airway inflammation). Therefore, this wide exposure of NEB could readily modify or interfere with their normal function (Fig. 6).

Since NEB appear prominent in human fetal/neonatal lungs, early studies of pathological material focused on disorders of prematurity and

abnormal lung development. These studies were reviewed recently (Cutz et al., 1995; Cutz, 1997) and therefore only the relevant disorders will be discussed here. In acute lung injury in an immature lung (hyaline membrane disease, HMD), reduced number of NEB was reported, whereas marked hyperplasia of NEB has been described in lungs of infants with bronchopulmonary dysplasia (BPD)—a form of chronic lung disease in premature infants (Cutz et al., 1984; Johnson et al., 1985). Although the precise mechanism for these changes is not known, in HMD, acute hypoxia causing NEB cell degranulation and or direct epithelial injury is thought to be responsible for the decrease in NEB cells. In BPD, the etiology is likely complex as multiple factors such as chronic hypoxia due to lung tissue damage, inflammation and repair process, as well as the use of supplemental oxygen contribute to disease progression. Hyperplasia of NEB and the associated increase in amine and peptide mediators has been linked to some of the clinical manifestations, i.e. pulmonary hypertension, hyper reactive airways and increased apnea episodes (Johnson and Georgieff, 1989).

Hyperplasia of NEB associated with dysfunction of peripheral chemoreceptors has been recently proposed as a potential mechanism for congenital central hypoventilation syndrome (CCHS) (Cutz et al., 1997) and for sudden infant death syndrome (SIDS) (Perrin et al., 1991). We have reported two cases of CCHS with marked hypoplasia of carotid body glomus cells accompanied by a two fold increase in the size and number of NEB. We postulated that in CCHS, NEB hyperplasia may be compensatory and indicates possible interaction between arterial and airway O_2 sensors. In cases of SIDS, NEBs appear hyperplastic compared to age matched control infants (Perrin et al., 1991). The hyperplasia and hypertrophy of NEB is particularly striking in small peripheral airways and at bronchiole-alveolar junctions. The etiology or mechanisms of NEB hyperplasia in lungs of SIDS victims is unknown, but effects of chronic hypoxia and/or developmental delay have been postulated as possible factors. Abnormalities in carotid body catecholamines has been previously reported in SIDS infants (Perrin et al., 1984), suggesting that these sensors may be compromised, particularly in their response to hypoxia. An additional mechanism may involve maternal smoking, since nicotine is known to affect NEB structure and function. Recent study on NEB in relation to SIDS found potentiation of NEB hyperplasia in lungs of SIDS infants whose mothers smoked during pregnancy (Cutz et al., 1996). Mild airway inflammation reported in up to 30% of SIDS cases could be another potential mechanism affecting the function of NEB and represent another potential triggering factor for SIDS. Local production of inflammatory cytokines and reactive oxygen intermediates, including H_2O_2, could alter the O_2 sensor on NEB cell membrane (Fig. 6). The failure to signal airway hypoxia could in turn initiate a chain of events leading to SIDS. In this context, a recent report by Parkins et al. (1998) on effects of mild airway hypoxia (as may occur at high altitude or during air flights) triggering apnea in a proportion of 'normal' infants would support this hypothesis. It is also of interest to note that both BPD and Wilson–Mikity Syndrome, another rare chronic lung disease of premature infants, are associated with hyperplasia of NEB and increased incidence of sudden unexpected death (Cutz et al., 1984).

6. Future prospects

Research on NEB over the past two decades has provided detailed information on their morphology, distribution and expression of various neuroendocrine markers, but their role in the control of respiration or other pulmonary homeostatic mechanisms is unknown. Current data strongly suggests that NEB function as airway chemoreceptors, based on the extensive similarities in the morphologic and physiologic characteristics between NEB and the well-established arterial chemoreceptors, the carotid bodies. Both airway and arterial chemoreceptors sense O_2 through ion channel modulation, utilize a Ca^{2+}-dependent mechanism for stimulus-secretion coupling and are innervated by afferent (sensory) nerve endings. Recent development of the fresh lung slice technique should facilitate and enhance

future studies to locate NEB cell-derived nerve projections and ascertain NEB function in the intact, developing animal. Of particular interest would be neural tracing studies using horse radish peroxidase or recently developed fluorescent labeled probes. Whole animal studies could test the effects of oxidase inhibitors (i.e. DPI), or other specific blockers of the O_2 sensor molecular complex on postulated NEB functions, including control of respiration. A recently developed technique of immunotoxin targeting using antibodies against specific cell surface molecules could be applied to NEB ablation studies. The disruption of NEB function during critical stages of lung development or during neonatal adaptation could provide answers as to their precise homeostatic role. Furthermore, the recent introduction of a transgenic mouse model with a homozygous deletion of a NADPH oxidase subunit (Pollack et al., 1995) provides a unique opportunity to study the role of the oxidase in O_2 sensing. It should also be pointed out that additional mechanisms for O_2 sensing, not dependent on the oxidase, have been recently proposed (Peers, 1997). Undoubtedly, future studies will define their role and relative importance in O_2 sensing.

Another promising area of physiologic studies would be an investigation of the interaction between NEB as representative airway sensors and carotid bodies as arterial chemoreceptors. Such interaction may be most relevant during the perinatal period when NEB are most numerous and when carotid body function has not yet fully matured. It is conceivable that during the period of neonatal adaptation NEB may replace or complement carotid body function. Since NEB are directly exposed to inspired air they may be better suited to rapidly signal changes in O_2 concentration in advance of the carotid body.

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K^+ and Ca^{2+} channel activity and cytosolic [Ca^{2+}] in oxygen-sensing tissues

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Abstract

Ion channels are known to participate in the secretory or mechanical responses of chemoreceptor cells to changes in oxygen tension (P_{O_2}). We review here the modifications of K^+ and Ca^{2+} channel activity and the resulting changes in cytosolic [Ca^{2+}] induced by low P_{O_2} in glomus cells and arterial smooth muscle which are well known examples of O_2-sensitive cells. Glomus cells of the carotid body behave as presynaptic-like elements where hypoxia produces a reduction of K^+ conductance leading to enhanced membrane excitability, Ca^{2+} entry and release of dopamine and other neurotransmitters. In arterial myocytes, hypoxia can inhibit or potentiate Ca^{2+} channel activity, thus regulating cytosolic [Ca^{2+}] and contraction. Ca^{2+} channel inhibition is observed in systemic myocytes and most conduit pulmonary myocytes, whereas potentiation is seen in a population of resistance pulmonary myocytes. The mechanism whereby O_2 modulates ion channel activity could depend on either the direct allosteric modulation by O_2-sensing molecules or redox modification by reactive chemical species.

Keywords: Carotid body, ion channels; Control of breathing, carotid body; Ion channels, K^+, Ca^{2+}; Smooth muscle, vascular, ion channels

1. Introduction

Oxygen-sensing is a general phenomenon that allows cells to adapt to stressing hypoxic environments. In most cell types, protracted hypoxia leads to alteration of metabolic function and the

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expression of genes encoding enzymes, hormones and growth factors (for a review see Bunn and Poyton, 1996). However, there are cells able to respond to decrements in O_2 tension (P_{O_2}) in the course of seconds or minutes with changes in their excitability, contractility or secretory activity. These fast-responsive, O_2-sensitive cells are normally located in chemoreceptor organs (i.e. the carotid, aortic and neuroepithelial cell bodies, neonatal adrenal medulla or vascular smooth muscle) that participate in cardiovascular and ventilatory control (for reviews see López-Barneo and Weir, 1998). Besides their physiological significance, O_2-sensitive chemoreceptors have also a growing clinical interest because they appear to be involved in the pathogenesis of several diseases such as sleep apnea, sudden infant death syndrome or pulmonary hypertension (Cutz et al., 1997; Weir et al., 1998). Over the past decade it has become well established that modulation of ion channel activity by changes in P_{O_2} participates in the secretory or mechanical responses of chemoreceptor cells to low P_{O_2} (see López-Barneo, 1996; López-Barneo et al., 1998). O_2-regulated K^+ channels, initially observed in glomus cells of the carotid body, are present in a variety of tissues including pulmonary vascular smooth muscle, neuroepithelial cells in the airways, chromaffin cells and central neurons. Recent work has shown that in vascular myocytes or neurons Ca^{2+} channel activity can be under control of P_{O_2}, and there are reports indicating that in some preparations Na^+ and Cl^- channels are also altered by hypoxia. In this article we briefly describe the properties of K^+ and Ca^{2+} channels in O_2-sensing tissues, emphasizing the work done in our laboratory on carotid body and vascular smooth muscle cells as representative examples of O_2-sensitive neurosecretory and contractile systems, respectively.

2. Modulation of K^+ channels by O_2 tension

The first clues suggesting the existence of ion channels regulated by O_2 tension came from work in dispersed glomus cells of the carotid body showing that, at least in rabbits, they are electrically excitable (Duchen et al., 1988; López-Barneo et al., 1988) and have a K^+ current inhibited by hypoxia (López-Barneo et al., 1988; Delpiano and Hescheler, 1989; Peers, 1990; Stea and Nurse, 1991; Chou and Shirahata, 1996; for additional references see González et al., 1994; López-Barneo et al., 1998). An example of O_2-sensitive K^+ current is illustrated in Fig. 1A with recordings from a patch-clamped rabbit glomus cell during depolarizing pulses to +20 mV and exposed to normoxic ($P_{O_2} \approx 150$ Torr) and hypoxic ($P_{O_2} \approx 20$ Torr) solutions. In this experiment the inward current (mainly carried by Ca^{2+} ions) was unaffected by hypoxia but the outward K^+ current amplitude was reduced by about 25% on exposure to low P_{O_2}. These observations were followed by the biophysical characterization of the various types of voltage-gated K^+ channels existing in rabbit glomus cells and the identification of O_2-sensitive K^+ channels, expressed with a density of $\approx$1000–2000 per cell, as the major contributor to the whole-cell K^+ current (Ganfornina and López-Barneo, 1991, 1992). As shown

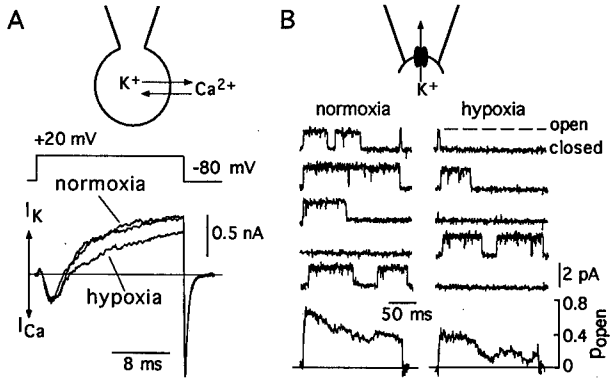

Fig. 1. Major O_2-dependent electrophysiological properties of rabbit glomus cells. (A) Macroscopic inward and outward currents of a glomus cell and reversible inhibition of the outward current by hypoxia ($P_{O_2} \approx 20$ Torr). Control and recovery traces in normoxia ($P_{O_2} \approx 150$ Torr) are shown superimposed. Tetrodotoxin was added to the external solution to block Na^+ conductance. (B) Single-channel recordings from an excised membrane patch containing at most one open O_2-sensitive K^+ channel. Depolarizing pulses applied from −80 to +20 mV. Ensemble averages indicating the single-channel open probability in normoxia and hypoxia are from 15 and 22 successive recordings in the two experimental conditions. (Modified from Ganfornina and López-Barneo, 1992; Montoro et al., 1996.)

in Fig. 1B, hypoxia decreases single-channel open probability (between 20 and 40%) but leaves unaltered the single-channel conductance (about 20 pS in physiological extra and intracellular K^+ concentrations). Although inhibition of K^+ conductance by hypoxia is a common feature of glomus cells, the type of K^+ channel regulated by O_2 varies in the different mammalian species and experimental conditions. In cat glomus cells the O_2-sensitive K^+ current has some characteristics similar to the current in the rabbit (Chou and Shirahata, 1996) but in neonatal and adult rats Ca^{2+}-dependent, maxi-K^+ channels are the ones regulated by low P_{O_2} (Peers, 1990; López-López et al., 1997). It seems, however, that the type of rat K^+ channel influenced by hypoxia changes if animals are reared in low P_{O_2} environments (Wyatt et al., 1995). The O_2-sensitive channel retains similar activation and inactivation characteristics but looses its sensitivity to charybdotoxin and no longer contributes to resting potential. Macroscopic K^+ currents similar to those of the carotid body are reduced by hypoxia in neuroepithelial cells of the lung airways (Youngson et al., 1993) as well as in adrenomedullary (Thompson et al., 1997) and PC12 (Zhu et al., 1996) cells. In this cell line the expression of Kv1.2 channels is up-regulated by low P_{O_2} and these channels appear to be those O_2-sensitive (Conforti and Millhorn, 1997). Besides the voltage-dependent O_2-sensitive K^+ channels, it has been reported in rat glomus cells a leak K^+ conductance which seems to mediate the depolarizing receptor potential triggered by low P_{O_2} (Buckler, 1997). We have observed in some rabbit glomus cells exposed to hypoxia changes in the holding current compatible with inhibition of an O_2-sensitive leak K^+ current similar to that in the rat. However in other cells the holding current is either unaffected by hypoxia or varies in the opposite direction. Interestingly, in some of the cells exhibiting O_2-dependent changes in the holding current the voltage-dependent K^+ currents are unaltered by low P_{O_2}. Hypoxic inhibition of voltage-dependent K^+ channels favours the increase of action potential firing frequency in spontaneously active rabbit glomus cells (Montoro et al., 1996), but in quiescent cells (such as those in the rat carotid body lacking Na^+ channels and with reduced ability of spontaneous pacemaking activity) the leak O_2-sensitive channels may have an important role in the initiation of the response to low P_{O_2}.

Regardless of the K^+ channel type modulated by P_{O_2}, the consequence of exposure to hypoxia in glomus and chromaffin-like cells is the reduction of K^+ conductance and an increase of cellular excitability leading to the opening of Ca^{2+} channels, Ca^{2+} influx and elevation of cytosolic [Ca^{2+}] (López-Barneo et al., 1993; Buckler and Vaughan-Jones, 1994; Ureña et al., 1994; Zhu et al., 1996; Zhong et al., 1997). The relationship between cytosolic [Ca^{2+}] and ambient P_{O_2} in rabbit glomus cells is illustrated in Fig. 2. Application of graded hypoxia to the cells results in parallel elevations of cytosolic [Ca^{2+}] (Fig. 2A). Fig. 2B shows that the relation between cytosolic Ca^{2+} and P_{O_2} follows an hyperbolic function (see below). Fig. 2C and D demonstrate that suppression of Ca^{2+} currents by removal of external Ca^{2+} or blockade of the channels with Cd^{2+}, reversibly abolishes the rise of cytosolic Ca^{2+} evoked by low P_{O_2}. Since glomus cells were known to contain large amounts of dopamine and other transmitters (see Fidone and González, 1986) we have characterized the neurosecretory response to hypoxia in single cells by the amperometric detection of the released oxidizable substances (Ureña et al., 1994). Application of a polarized carbon fiber near the surface of a glomus cells permits the identification of secretory spikes representing discrete events due to release of single vesicles (Fig. 3, top). Typical exocytotic responses to hypoxia are shown in Fig. 3 (bottom), where it is also illustrated that the low P_{O_2}-induced transmitter release requires extracellular Ca^{2+}. Thus, glomus cells behave as O_2-sensitive presynaptic-like elements with a secretory response to hypoxia that is almost absolutely dependent on the influx of extracellular Ca^{2+}. The resemblance between the O_2-sensitive responses at the cellular and organ levels indicates that the neurosecretory properties of single glomus cells are major contributors to the chemosensory function of the carotid body. For example, both in single cells and in whole carotid bodies, the hypoxic response is blocked by

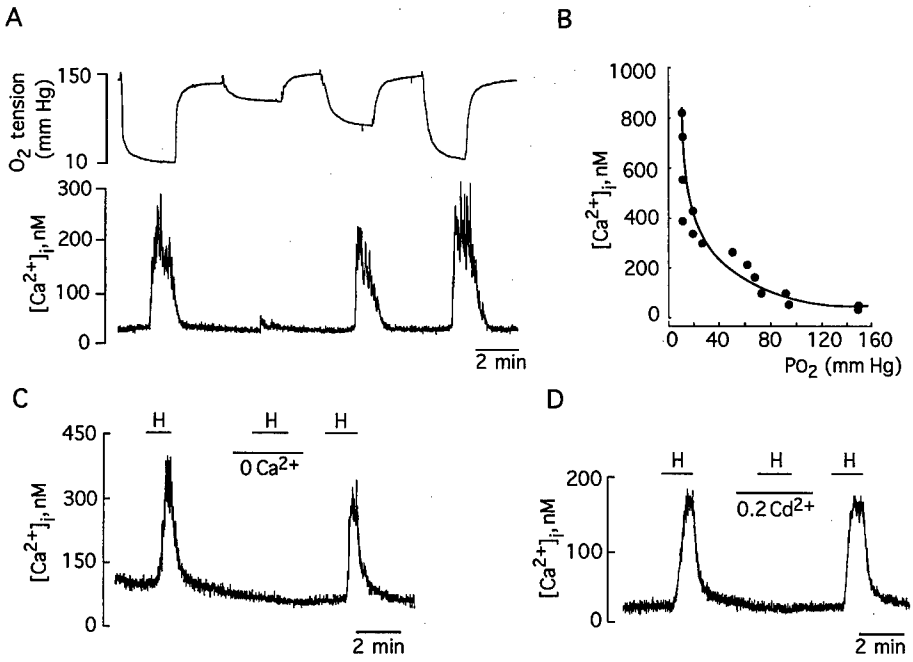

Fig. 2. Rise of cytosolic Ca^{2+} in glomus cells in response to low P_{O_2}. (A) Gradual elevations of cytosolic $[Ca^{2+}]$ in a fura-2-loaded cell in response to various levels of hypoxia. The signal from an O_2-sensitive electrode is shown at the top. (B) Relationship between cytosolic $[Ca^{2+}]$ and P_{O_2} in several rabbit glomus cells. (C, D) Abolishment of the hypoxia-induced rise of cytosolic Ca^{2+} after removal of extracellular Ca^{2+} or blockade of Ca^{2+} channels with 0.2 mM Cd^{2+}. (Modified from Ureña et al., 1994; Montoro et al., 1996.)

Ca^{2+} channel antagonists. In addition, the hyperbolic relationship between cytosolic Ca^{2+} or secretory rate and P_{O_2} in single cells is almost superimposable to the curve relating the changes in afferent sensory discharges as a function of O_2 tension in either isolated or in situ carotid bodies. However, whether or not dopamine release is absolutely required for the chemosensory function of the carotid body is still open to discussion since the nature of the transmitter that activates the afferent fibers of the sinus nerve is unknown. The actual role of dopamine might be autocrine, as feedback regulator of glomus cell secretory activity, rather than that of a direct transmitter molecule. It is known that exogenous dopamine inhibits the hypoxic chemosensory discharges in afferent nerve fibers (Donnelly et al., 1981) and we have shown that in dispersed glomus cells application of dopamine can selectively reduce the amplitude of Ca^{2+} currents (Benot and López-Barneo, 1990).

O_2-sensitive K^+ channels have been also studied in some central neurons (Jiang and Haddad, 1994) and, in more detail, in smooth muscle cells of the pulmonary arterial tree (Post et al., 1992; Yuan et al., 1993; Osipenko et al., 1997). In myocytes dispersed from resistance pulmonary vessels inhibition of voltage-dependent K^+ channels by low P_{O_2} causes membrane depolarization, opening of L-type Ca^{2+} channels and contraction. These phenomena contribute to explain hypoxic pulmonary vasoconstriction, a response specific of vessels in the pulmonary circulation which helps to maintain high resistance in fetal lung and in the adult contributes to the matching of ventilation and perfusion by diverting blood to the better ventilated alveoli (see Weir et al., 1998). As it occurs in the neurosecretory systems, the type of O_2-sensitive K^+ channel in the resistance vessels of the pulmonary circulation might change with age or animal species. In sheep fetuses the O_2-sensitive channel has characteristics of the

Ca^{2+}-dependent maxi-K^+ channels (Cornfield et al., 1996) but in young and adult animals the channel modulated by hypoxia is of the delayed rectifier type (Yuan et al., 1993; Archer et al., 1996). The molecular nature of the O_2-regulated K^+ channel in vascular myocytes is unknown although it has been shown that chronic hypoxia inhibits selectively the expression of Kv1.2 and Kv1.5 channels in pulmonary vascular smooth muscle cells (Wang et al., 1997). In most O_2-sensitive cells studied so far the K^+ conductance is reduced by hypoxia but the opposite effect (potentiation of K^+ current by low P_{O_2}) has been also described. In sheep ductus arteriosus there are K^+ channels potentiated by low P_{O_2} which are inhibited in normoxic conditions. Reduction of the K^+ current after birth may contribute to the closure of the ductus thus preventing in the newborn the mixture of oxygenated and deoxygenated blood (Tristani-Firouzi et al., 1996).

3. Ca^{2+} channel activity and cellular responses to hypoxia

As discussed in the previous section, it is well-established that changes in transmembrane Ca^{2+} influx are ultimately necessary for the secretory or mechanical responses to hypoxia in O_2-sensitive tissues. These modifications appear to result from primary alterations in the function of O_2-sensitive K^+ channels which regulate membrane potential and, thus, the activity of voltage-dependent Ca^{2+} channels. However, there are recent indications suggesting that Ca^{2+} channels may be also directly influenced by changes in O_2 tension (Franco-Obregón et al., 1995; Franco-Obregón and López-Barneo, 1996; Soloviev et al., 1996; Miranov and Richter, 1998). Our initial experimental observations suggesting the modulation of Ca^{2+} channels by O_2 tension were done in dispersed myocytes from systemic (femoral, celiac and mesenteric) and coronary arteries. These

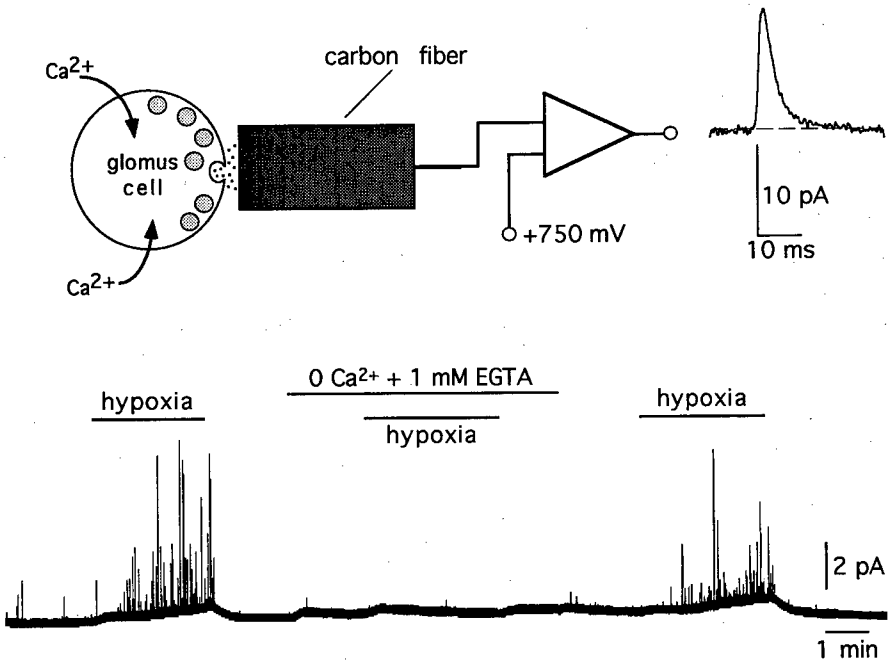

Fig. 3. Secretory response of a single glomus cell to low P_{O_2}. As indicated by the diagram at the top, dopamine release was monitored by amperometry with an 8 μm polarized (to +750 mV) carbon fiber electrode placed near the cell and quantal secretory events appeared as spike-like activity representing the fusion of individual secretory vesicles. The trace at the bottom indicates that the hypoxia-induced secretory activity was abolished by removal and chelation of extracellular Ca^{2+}.

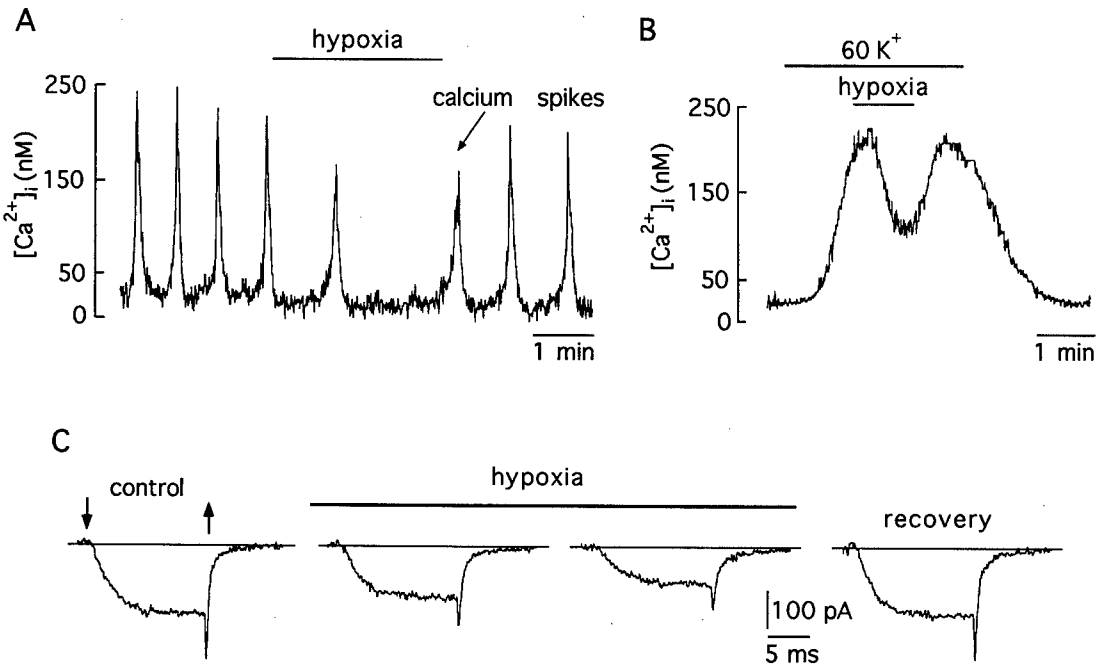

Fig. 4. Alteration of Ca^{2+} homeostasis in vascular myocytes by hypoxia. (A) Recording of cytosolic $[Ca^{2+}]$ in a fura-2-loaded dispersed rabbit celiac myocyte illustrating the reversible inhibition of Ca^{2+} oscillations in response to hypoxia. (B) Reversible reduction of cytosolic $[Ca^{2+}]$ in a pig coronary myocyte in which membrane voltage-dependent Ca^{2+} channels were activated by depolarization with high extracellular K^+. (C) Calcium currents recorded from a rabbit celiac myocyte during 15-msec step depolarizations to +10 mV from a holding potential of −80 mV. After exposure to hypoxia ($P_{O_2} \approx 20$ Torr) there is a progressive inhibition of current amplitude. Reversibility is illustrated by the recovery trace. (Modified from Franco-Obregón et al., 1995, 1998).

preparations are known to dilate in response to local decrements in P_{O_2} by means of several mechanisms, some of them independent of endothelial integrity (see Franco-Obregón et al., 1998). Dispersed systemic myocytes loaded with fura-2 can exhibit spontaneous oscillations of cytosolic $[Ca^{2+}]$ (Ca^{2+} spikes) due to release of the cation from internal stores. Hypoxia produces a reversible decrease in basal Ca^{2+} and reduction in frequency, or even suppression, of the Ca^{2+} oscillations (Fig. 4A). Similar low P_{O_2}-dependent reductions in cytosolic Ca^{2+} are also observed in myocytes that do not have spontaneous Ca^{2+} spikes but where cytosolic $[Ca^{2+}]$ is increased by depolarization with high external K^+ (Fig. 4B). ATP regulated K^+ (K_{ATP}) channels in myocytes are known to be major regulators of arterial tone in conditions of strong and protracted exposure to low P_{O_2} since their opening leads to membrane hyperpolarization and smooth muscle relaxation (Daut et al., 1990). However, there are several facts indicating that activation of these channels cannot fully account for the responses to hypoxia described above. Low P_{O_2}-induced reductions in cytosolic $[Ca^{2+}]$ are seen with mild hypoxia without compromise of cell's respiration and in the presence of glibenclamide, a blocker of K_{ATP} channels (Franco-Obregón et al., 1998). Moreover, hypoxia can also relax arterial rings after K^+-evoked contractures (Marriott and Marshall, 1990) and decreases cytosolic Ca^{2+} in cells bathed in high external K^+ (Fig. 4B and lower left panel in Fig. 6; see also Vadula et al., 1993), a condition at which opening of K_{ATP} channels would favour cell depolarization to a voltage near the K^+ equilibrium potential and Ca^{2+} influx. Modulation of Ca^{2+} channels by changes in O_2 tension can be evidenced in patch-clamped myocytes where exposure to hypoxia leads to a gradual and reversible reduction in the amplitude of macro-

scopic Ca^{2+} currents (Fig. 4C). Besides in arterial myocytes, inhibition of Ca^{2+} currents by hypoxia has been described in other types of smooth muscle cells, in glomus cells at potentials near threshold and in central neurones (for review see López-Barneo et al., 1998). Interestingly, L-type Ca^{2+} channels resulting from the stable expression of the cardiac α1C subunit in HEK cells are also reversibly inhibited by lowering P_{O_2} (Fearon et al., 1997).

Given that hypoxic pulmonary vasoconstriction is mainly observed in fine branches of the pulmonary arterial tree whereas vasodilation is normally produced in the main pulmonary artery, we have tested whether low P_{O_2} has differential effects on Ca^{2+} currents and cytosolic $[Ca^{2+}]$ homeostasis in conduit and resistance myocytes. In patch-clamped smooth muscle cells dispersed from the main pulmonary arterial trunk (conduit myocytes) hypoxia produces a reduction of Ca^{2+}

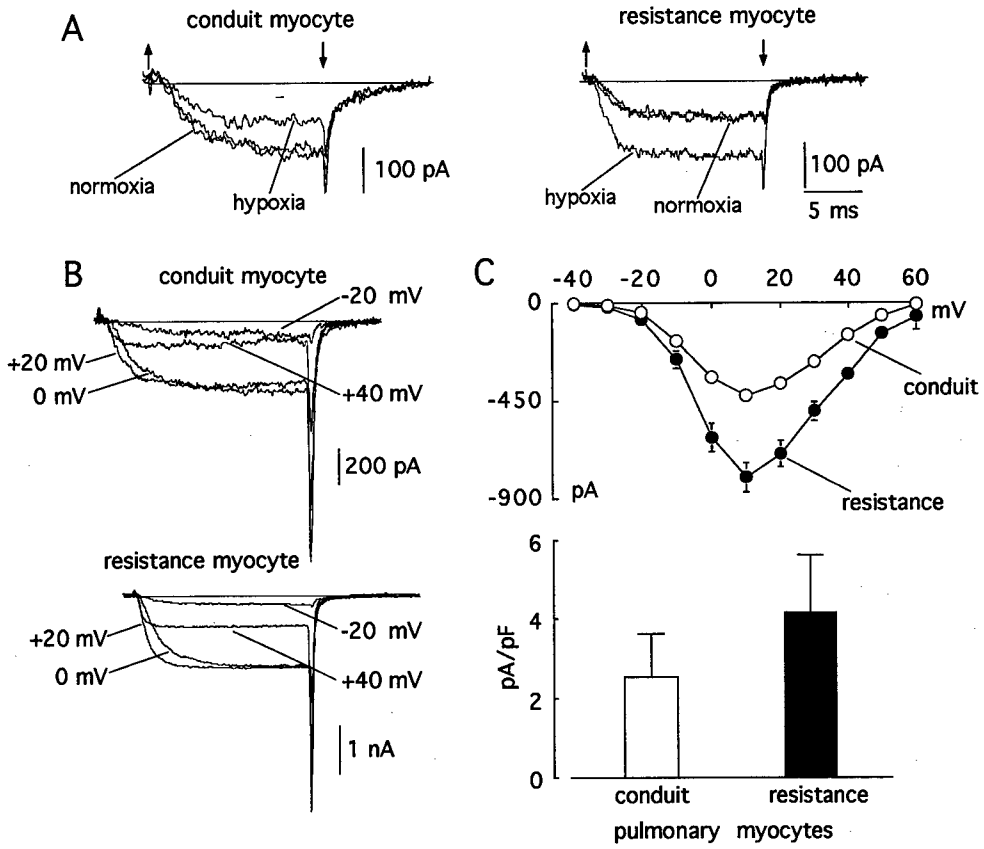

Fig. 5. Dual modulation of Ca^{2+} channel activity by low P_{O_2} in pulmonary arterial myocytes. (A) Currents recorded from patch-clamped cells dispersed from proximal (conduit) and distal (resistance) pulmonary arteries during depolarizations to −10 mV from a holding potential of −80 mV. The onset and end of the depolarizing pulses are indicated by the arrows. The control and recovery traces recorded in the normoxic solution are shown superimposed. (B) Families of Ca^{2+} currents recorded from representative conduit and resistance pulmonary myocytes during depolarizations to the indicated membrane potentials from a holding potential of −80 mV. (C) Top: mean calcium current–voltage relationships in conduit (open circles; n = 41) and resistance (filled circles; n = 21) myocytes of the pulmonary arterial tree. The plot shows the average peak inward current (ordinate, mean ± standard error) elicited with voltage pulses to the indicated membrane potentials (abscissa) from the holding potential of −80 mV. Despite the differences in magnitude, the shapes of the current–voltage curves are the same in the two classes of smooth muscle cells. (C) Bottom: current density in conduit and resistance myocytes. (Modified from Franco-Obregón and López-Barneo, 1996.)

current amplitude similar to the effect described before in systemic and coronary arteries (Fig. 5A, left). However, in a high percentage of cells dispersed from tertiary arterial branches (resistance myocytes) hypoxia produces a potentiation of the calcium current which is more apparent in currents elicited by depolarizations near threshold (Fig. 5A, right). Apart from the differential modulation by low P_{O_2}, Ca^{2+} currents appear to be similar in the two classes of myocytes as indicated by their time course and current-voltage relationship (Fig. 5B and C). Interestingly, Ca^{2+} current density in resistance myocytes is about 2-fold the value in conduit myocytes (Fig. 5C), which might help to maintain a high vasomotor tone in the peripheral branches of the pulmonary arterial tree. The opposite effects of low P_{O_2} on Ca^{2+} channel activity in conduit and resistance myocytes are paralleled by differential responses of cytosolic Ca^{2+} to hypoxia in the two types of cells (Fig. 6). Measurements of cytosolic Ca^{2+} in both conduit and resistance pulmonary myocytes reveal the existence of oscillations of cytosolic Ca^{2+}, or Ca^{2+} spikes, much like those previously described in cells of the systemic vessels (Ureña et al., 1996). The Ca^{2+} spikes in some conduit and resistance pulmonary myocytes are modulated by changes in bathing P_{O_2}, although in diametrically opposed manners. In the majority of conduit myocytes a reduction in bathing P_{O_2} induces a drop in basal cytosolic Ca^{2+} concentration and a decrease, or complete supression, in the Ca^{2+} spike frequency. These changes are generally associated with an increase in Ca^{2+} spike amplitude (Fig. 6, left, top). Although this response to hypoxia is also seen in some myocytes isolated from fine arterial branches, in more than 50% of the resistance myocytes low P_{O_2} induces the opposite effect: increase in basal cytosolic [Ca^{2+}] accompanied by a decrement in Ca^{2+} spike amplitude (Fig. 6, right, top). In conduit myocytes the drop in P_{O_2} mimics the effects of removal of extracellular Ca^{2+} (Fig. 6, left, center) and can counteract the increment of basal cytosolic [Ca^{2+}] and reduction of Ca^{2+} spike amplitude observed with elevated extracellular K^+ (Fig. 6, left, bottom). In contrast, the response to hypoxia of resistance myocytes resembles the effect of high external K^+ (Fig. 6, right, center) and can be reversibly counteracted by selectively blocking the activity of L-type Ca^{2+} channels with nifedipine (0.5 μM) (Fig. 6, right, bottom). These results strongly suggest that low P_{O_2} modulates the activity of Ca^{2+} channels in opposed manners in different regions of the pulmonary arterial tree. In myocytes of conduit arteries hypoxia leads to inhibition of the Ca^{2+} channels, whereas it can potentiate or inhibit Ca^{2+} channel activity in different subpopulations of resistance myocytes. These results also agree with previous work showing that the development of hypoxic pulmonary vasoconstriction in the distal regions of the pulmonary artery can be either inhibited or enhanced with L-type Ca^{2+} channel antagonist or agonist, respectively (see Franco-Obregón et al., 1998). Potentiation of L-type Ca^{2+} channel activity by low P_{O_2} may not be specific of resistance pulmonary myocytes since a similar observation has been recently reported in inspiratory neurons (Miranov and Richter, 1998).

4. Oxygen-sensitivity of ion channels

The progress in research in recent years has produced numerous observations describing new O_2-regulated channels and/or cellular phenomena where they may play a role. However, the molecular nature of the mechanisms underlying O_2-sensing by ion channels remain essentially unknown. Our own experience with carotid body and vascular smooth muscle cells indicates that the O_2-sensitivity of ion channels is a rather labile process, easily altered by cell dissociation procedures, which may depend on subtle physico-chemical changes difficult to detect and characterize. It is also unknown whether the O_2-dependent mechanisms that mediate ion channel regulation in fast O_2-responsive cells are similar to those operating in a broad variety of cells types where protracted hypoxia regulates gene expression (see Ratcliffe et al., 1998). In some preparations regulation of ion channels by P_{O_2} appears to be a membrane delimited mechanism since it is resistant to intracellular dialysis and can be observed in excised membrane patches (Ganfornina and López-Barneo, 1991; Ji-

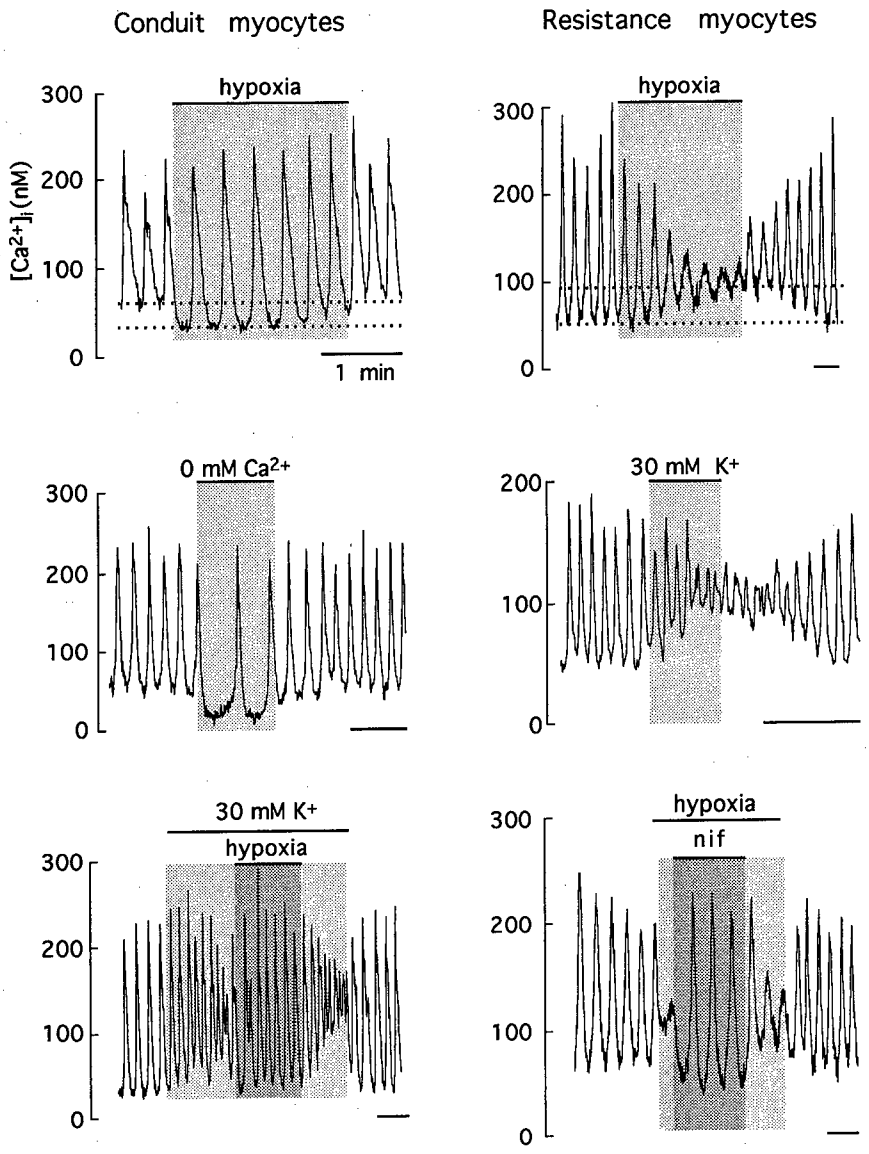

Fig. 6. Contrasting effects of hypoxia on the cytosolic $[Ca^{2+}]$ and Ca^{2+} oscillations (Ca^{2+} spikes) in conduit (left column) and resistance (right column) pulmonary myocytes. The shaded regions represent the times during which the bath solution was exchanged for the indicated experimental conditions. Left column: top, reduction of basal Ca^{2+} levels (dotted lines) and the frequency of the oscillations by low P_{O_2} in a representative conduit myocyte; center, removing extracellular Ca^{2+} from the bathing solution reversibly decreases basal cytosolic $[Ca^{2+}]$ and decreases the frequency of the Ca^{2+} spikes; bottom, increase of basal Ca^{2+} and the frequency of the oscillations by high external K^+. The effect of K^+ is counteracted by hypoxia. Right column: top, increase of basal Ca^{2+} (dotted lines) and reduction of the Ca^{2+} spikes amplitude during exposure to hypoxia in a resistance myocyte; center, depolarizing the myocytes with 30 mM extracellular K^+ increases basal cytosolic $[Ca^{2+}]$, spikes frequency and reduces spike amplitude; bottom, the effect of hypoxia is counteracted by blockade of L-type Ca^{2+} channels with nifedipine (0.5 μM). The P_{O_2} of the solutions were ≈150 (normoxia) and 20–30 (hypoxia) Torr. All calibration bars indicate 1 min. (Modified from Ureña et al., 1996.)

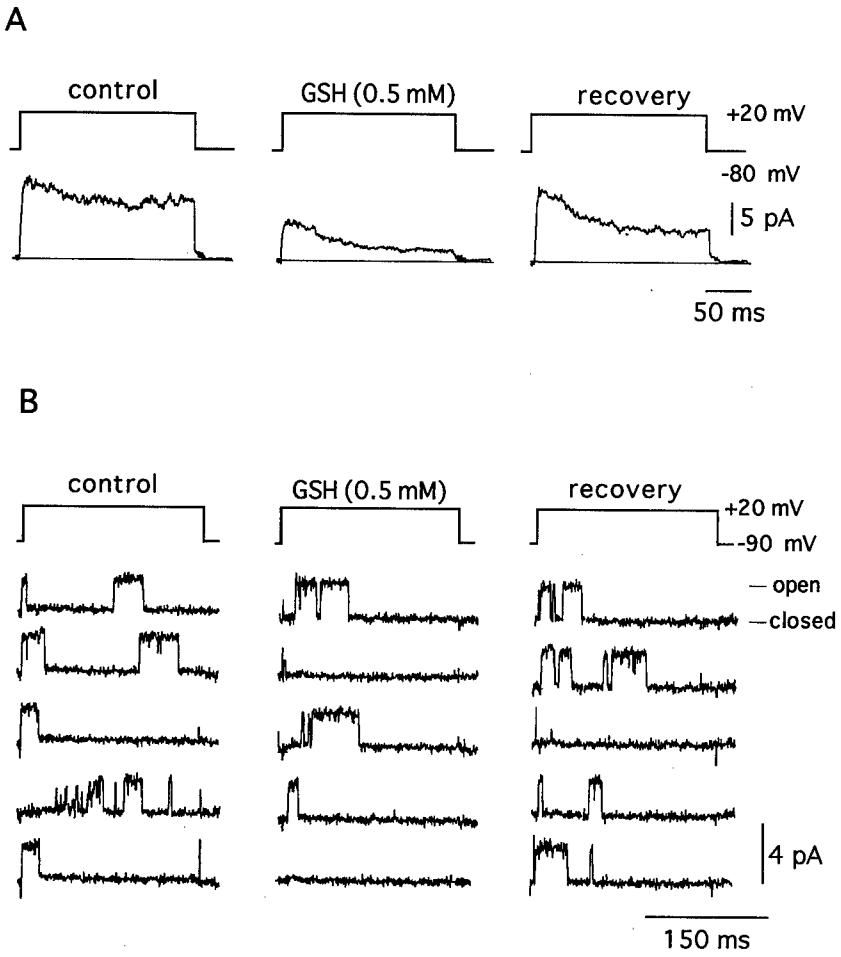

Fig. 7. Redox regulation of the O_2-sensitive K^+ channels of rabbit glomus cells. (A) Currents recorded from an inside-out macropatch during depolarizing pulses to +20 mV in the standard solution (control) and after addition of reduced glutathione (GSH, 0.5 mM) to the bath. GSH reversibly reduced current amplitude. (B) Modulation of a single O_2-regulated K^+ channel by the application of reduced glutathione (GSH, 0.5 mM) to the internal face of the membrane. The recordings were obtained from an inside-out membrane patch with a functional channel during depolarization to +20 mV. The concentrations of K^+ were 2.7 and 140 mM in the external and internal solutions, respectively. (Modified from Benot et al., 1993; López-Barneo et al., 1998.)

ang and Haddad, 1994). Therefore, it is conceivable that there are O_2 sensors associated with the ion channels capable of undergoing conformational changes during oxygenation and deoxygenation and, thus, modifying allosterically the channel's kinetic properties. The best candidates for this kind of regulation are membrane bound heme proteins such as the cloned O_2 sensor of *Rhizobium meliloti* (Fix L) that can change activity according to its oxygenated state (see Bunn and Poyton, 1996). Interestingly, the oxygenation state of hemoglobin, associated with the internal face of the membrane, is also known to alter the ion transport properties of erythrocytes (Motais et al., 1987; Lauf, 1998). Another possibility to consider is the existence of structural domains in some of the main (α or β) channel subunits that can bind reversibly molecular oxygen and modify their function. In this respect, it is important to mention that there are several reports describing the modulation by P_{O_2} of recombinant K^+ and Ca^{2+} channels expressed in heterologous systems,

although the precise molecular mechanisms involved have remained unexplained so far (Ortega-Sáenz et al., 1996; Fearon et al., 1997; López-Barneo et al., 1997; Patel et al., 1997; Pérez-García et al., 1998).

Besides the direct effect of O_2 tension on ion channels, another possibility is the participation of redox based mechanisms in O_2-sensing. An attractive hypothesis is that the modifications of O_2 tension are linked to channel activity through variations in the concentration of oxidants or reductants which modify the redox state of thiol groups in the channel molecule (Acker et al., 1989; Archer et al., 1993; Benot et al., 1993). It has been shown in recombinant K^+ channels that the redox state of cysteine residues in the amino terminal of the α subunits or in some auxiliary β subunits regulates inactivation. Moreover, it is known that reductants, such as reduced glutathione (GSH) or dithiothreitol (DTT), can mimic the effect of hypoxia on the O_2-regulated K^+ channels (Archer et al., 1993; Benot et al., 1993; Yuan et al., 1994; López-Barneo et al., 1998). The redox regulation of K^+ conductance in rabbit glomus cells is illustrated in Fig. 7. The traces show the currents recorded from an inside-out macropatch with approximately 20 channels (A) and from a patch with a single functional channel (B). In both cases application of GSH to the internal face of the membrane produced reversible inhibition of channel activity. As it occurs during exposure to low P_{O_2}, GSH reduces channel open probability without affecting the single-channel conductance (López-Barneo et al., 1998). Hence, it is plausible that some of the P_{O_2}-dependent effects observed in the various O_2-sensitive K^+ channels studied are due to changes in the production of oxygen radicals which modify the redox state of the channel protein. In the cell types that exhibit a special sensitivity to P_{O_2} changes (for instance glomus and neuroepithelial cells or pulmonary arterial smooth muscle) it has been postulated the existence of specific membrane bound oxidases associated with the channel molecule and capable of generating the reactive species in the vicinity of the target residues. Among the proposed candidates are the oxidases similar to the NADPH oxidase of neutrophils or the family of cytochrome P-450 oxidases (Acker et al., 1989; Youngson et al., 1993; Yuan et al., 1995). The putative oxidase acting as O_2 sensor could be co-expressed with the main pore-forming α subunit, thus conferring to the channels O_2-sensitivity.

In conclusion, O_2-regulated K^+ and Ca^{2+} ion channels are involved in the cellular responses to hypoxia. These channels, affected by changes in O_2 tension in the range of seconds or minutes, are present in fast O_2-responsive cells of chemoreceptor tissues or organs. Although the nature of the mechanisms underlying the interaction of O_2 and the channels is still unknown, several possible forms, which may well act in parallel, are discussed. O_2-sensing by ion channels and the O_2-dependent regulation of gene expression are possibly related phenomena, acting in different time ranges, that may share similar basic principles and mechanisms.

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RESPIRATION PHYSIOLOGY

Roles for NAD(P)H oxidases and reactive oxygen species in vascular oxygen sensing mechanisms

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Abstract

Observations that physiological levels of O_2 control the rates of production of reactive O_2 species by systems including NAD(P)H oxidases and that certain of these species have signalling mechanisms that regulate vascular tone has resulted in consideration of these systems in processes that mediate the sensing of changes in P_{O_2}. Evidence exists for the participation of hydrogen peroxide-dependent regulation of prostaglandin production and soluble guanylate cyclase activity, resulting from the metabolism of peroxide by cyclooxygenase and catalase, respectively, in P_{O_2}-elicited signalling mechanisms that regulate vascular force generation. A microsomal NADH oxidase whose activity is controlled by the redox status of cytosolic NAD(H) appears to function as a P_{O_2} sensor in bovine pulmonary and coronary arteries where changes in O_2 levels control the production of superoxide anion-derived hydrogen peroxide and a cGMP-mediated relaxation response. Interactions with nitric oxide and superoxide anion, and the activity of glutathione peroxidase appear to influence the function of these O_2 sensing systems, and some of these interactions, along with the activation of other oxidases, may contribute to alterations in P_{O_2} sensing mechanisms under pathophysiological conditions that affect vascular function.

Keywords: Oxygen; Sensing; NADH oxidase; Peroxide; Signalling mechanisms; Reactive oxygen; Regulation of vascular tone; Smooth muscle; Vascular; Reactive oxygen

1. Introduction

In this review consideration is given to potential roles for signalling mechanisms involving reactive O_2 species derived from NAD(P)H oxidases in responses of vascular tissue to changes in O_2 tension. A large number of studies during the past

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40 years have provided evidence that vascular preparations show alterations in contractile function over a wide range of O_2 tensions that are observed in physiological systems. While mitochondrial regulation by changes in P_{O_2} has the potential to be a vascular O_2 sensor, there is substantial evidence for the existence of other O_2 sensing mechanisms in vascular tissue (Coburn, 1977). Much of the evidence used to exclude a role for a P_{O_2}-dependent modulation of mitochondrial metabolism in O_2-elicited vascular responses originates from the minor effects of mitochondrial inhibitors on these responses and from the knowledge that the O_2 requirements for mitochondrial metabolic function are saturated at O_2 levels of 1–2 μM (or at a $P_{O_2} > 1$ torr).

Early observations on some of the potential mechanisms of P_{O_2}-elicited vascular responses included evidence for changes in the production of prostaglandins (Kalsner, 1977; Roberts et al., 1981) and cGMP levels (Clyman et al., 1975). Reactive O_2 species merit consideration as mediators of O_2-elicited vascular responses since their formation from microsomal (Boveris et al., 1972) and mitochondrial (Boveris and Chance, 1973) sources appear to be regulated by physiological P_{O_2} levels that are known to influence vascular force and organ blood flow. Studies in hepatic tissue resulted in the suggestion by Helmut Sies (1977) that peroxide metabolism could potentially have a role in O_2 sensing through its metabolic influence on the peroxidatic reactions of enzymes including catalase and glutathione (GSH) peroxidase, an enzyme which could regulate cellular processes through alterations in GSH redox. Based on observations that reactive O_2 species were vasoactive and appeared to have distinct signalling mechanisms, it was suggested that these species could function in vascular O_2 sensing mechanisms that mediated responses to acute changes in P_{O_2} (Archer et al., 1986; Burke and Wolin, 1987). Some of the initial processes involving a role for reactive O_2 species that were considered as potential signalling mechanisms in P_{O_2}-elicited vascular responses included proposed roles for: (1) peroxide metabolism by cyclooxygenase (Wolin et al., 1990), an enzyme which is activated by peroxides to produce prostaglandins (Hemler et al., 1979); (2) peroxide metabolism by catalase stimulating the production of cGMP by activation of soluble guanylate cyclase (sGC) (Burke and Wolin, 1987; Burke-Wolin and Wolin, 1989); and (3) by effects of reactive O_2 species (radicals and peroxides) on membrane potential resulting from redox modulation of potassium channels (as a result of changes in the redox state of freely diffusible sulfhydryls, i.e. GSH) (Archer et al., 1986).

2. Signalling mechanisms involving reactive O_2 species

2.1. Eicosanoids

Initial observations in studies on the mechanism of action of cyclooxygenase resulted in the identification of a requirement for peroxides for the production of prostaglandin H_2 from arachidonic acid (Hemler et al., 1979). While the properties of the P_{O_2} dependence of the cyclooxygenase reaction remain rather poorly characterized, several early studies demonstrated that prostaglandins were mediators of P_{O_2}-elicited vascular responses (Kalsner, 1977; Roberts et al., 1981; Wolin et al., 1990; Omar et al., 1992). It has also been suggested that the metabolism of arachidonic acid by lipoxygenase (Voelkel, 1986) and cytochrome P450 (Harder et al., 1996) could also produce eicosanoid mediators of P_{O_2}-elicited vascular responses. Our current understanding suggests that several P_{O_2}-linked processes shown in Fig. 1 could be involved in the mechanisms that control the biosynthesis of prostaglandins and other eicosanoid mediators. In addition to the substrate requirements for O_2 of the arachidonic acid metabolizing enzymes involved in the oxygenation of arachidonic acid, the metabolism of peroxide by cyclooxygenase appears to convert the heme of this enzyme to an oxidation state that is required for initiating the metabolism of arachidonic acid (Hemler et al., 1979). The activities of the arachidonic acid metabolizing enzymes are also dependent on the release and availability of their substrate arachidonic acid. Arachidonic acid release by phospholipase A_2 seems to be stimu-

lated by peroxides. The activity of a cytosolic high molecular weight phospholipase A_2 appears to be controlled by signalling mechanisms regulated by peroxide, including phosphorylation through both protein kinase C and mitogen activated protein kinase pathways (Rao et al., 1995). Thus, modulation by P_{O_2} of the production of reactive O_2 species could potentially be an important process in mechanisms that control the release of arachidonic acid and the production of vasoactive cyclooxygenase and eicosanoid mediators of P_{O_2}-elicited responses.

2.2. Soluble guanylate cyclase and cGMP

The initial observation that changes in P_{O_2} could alter vascular tissue levels of cGMP was made in umbilical arteries in 1975, when cGMP was thought to be a mediator of vascular contraction (Clyman et al., 1975). Both reactive O_2 species, including peroxides and hydroxyl radical (Mittal and Murad, 1977; White et al., 1986) and nitric oxide (NO), were then proposed to participate in stimulation of the activity of the cytosolic or soluble form of guanylate cyclase (sGC). It is now known that cGMP is an intracellular regulator of vascular relaxation and that both exogenous and endogenously produced NO and H_2O_2 promote vascular relaxation through stimulation of the activity of sGC and the production of cGMP (Ignarro, 1989; Wolin, 1996). Both NO and H_2O_2 are mediators whose production requires O_2, and the usage of O_2 as a substrate for the production of these mediators provides a mechanism through which P_{O_2} can regulate changes in cGMP. Further investigation of how reactive O_2 species could control the activity of sGC uncovered several potential mechanisms. The activation of sGC by H_2O_2 in some vascular preparations appears to be mediated by H_2O_2 stimulating the production of NO by NO synthase (Furchgott, 1991) and by a mechanism involving the metabolism of peroxide by catalase (Burke and Wolin, 1987; Burke-Wolin and Wolin, 1990). Stimulation of sGC by catalase appears to occur at high picomolar to low nanomolar concentrations of H_2O_2, and it is associated with the formation of the Compound I species of catalase. The mechanism of relaxation to H_2O_2 is thought to be mediated through the metabolism of peroxide by catalase and stimulation of sGC. This relaxing mechanism is associated with increases in cGMP, and it appears to function under conditions where changes in GSH redox could not be detected (Wolin et al., 1996). Relaxation to H_2O_2 and the stimulation of sGC by H_2O_2 metabolism through catalase are attenuated by probes shown in Fig. 2 (Burke and Wolin, 1987; Burke-Wolin and Wolin, 1989, 1990; Cherry et al., 1990; Mohazzab-H. et al., 1996b, 1999). Probes which alter H_2O_2 metabolism by catalase include 3-amino-1,2,4-triazole (AT), ebselen and alcohols including ethanol and methanol. While the stimulation of sGC can be inhibited by methylene blue, LY83583 and O_2^-. Hydroxyl radical was initially proposed to cause stimulation of the activity of sGC (Mittal and Murad, 1977), however, evidence supporting this concept has not evolved, and more recently it has been suggested that hydroxyl radical is an inhibitor of the activation of vascular sGC (Kontos and Wei, 1993). An alternative interaction of reactive O_2 species is the inhibitory effect of O_2^- on the stimulation of sGC by either NO or H_2O_2. Thus, reactive O_2 species have several mechanisms through which they can control the activity of sGC and relaxation mediated by cGMP.

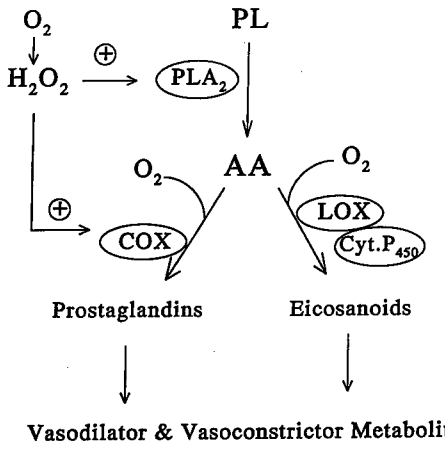

Fig. 1. Model for signalling mechanisms described in the text potentially involved in the sensing of changes in P_{O_2} associated with the regulation of prostaglandin and related eicosanoid mediators by reactive O_2 species. PL, phospholipids; PLA_2, phospholipase A_2; COX, cyclooxygenase; LOX, lipoxygenases; and Cyt P_{450}, cytochrome P_{450}.

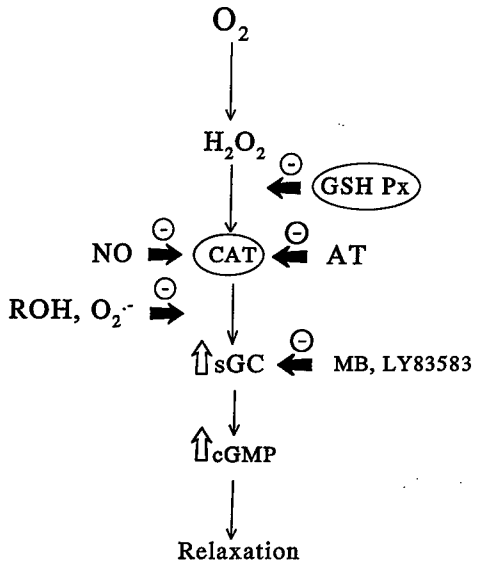

Fig. 2. Model for signalling mechanisms described in the text potentially involved in the sensing of changes in P_{O_2} associated with the regulation of soluble guanylate cyclase by reactive O_2 species. GSH Px, glutathione peroxidase; AT, 3-amino-1,2,4-triazole; ROH, alcohols including ethanol and methanol; MB, methylene blue; sGC, soluble guanylate cyclase.

2.3. Nitric oxide

Reactive O_2 species have several interactions with NO signalling which have the potential to participate in the expression of P_{O_2}-elicited vascular responses. It has been demonstrated that micromolar concentrations of H_2O_2 have the ability to stimulate the release of NO synthase-derived NO as a result of activation of the endothelium (Furchgott, 1991). The product of the interaction of NO with O_2^-, peroxynitrite ($ONOO^-$), may also be a participant in P_{O_2}-elicited responses. NO has also been observed to cause a prolonged inhibition of vascular catalase and relaxation to H_2O_2 (Mohazzab-H. et al., 1996b; Wolin et al., 1998). It appears that endogenous $ONOO^-$ formation is an important participant in the inhibition of cGMP-associated relaxation to H_2O_2 by NO since hypoxia and an intracellular scavenger of O_2^- inhibit this action of NO. However, it is not known if $ONOO^-$ directly inhibits catalase or if the NO subsequently generated as a result of $ONOO^-$ formation causes a tonic inhibition of this enzyme. Superoxide anion is an extremely potent antagonist of the actions of NO. Since the formation of O_2^- is also a P_{O_2}-dependent process, the interaction of NO with O_2^- could be a contributor to O_2-elicited responses. Mitochondrial respiration of both skeletal and cardiac muscle has been demonstrated to be reversibly inhibited by endothelium-derived NO (Shen et al., 1995; Xie and Wolin, 1996). The formation of $ONOO^-$ from the interaction of NO with O_2^- does not markedly alter the potency of NO as an inhibitor of respiration, but it converts it from a reversible process to an apparently irreversible action of NO (Xie and Wolin, 1996). Superoxide anion also appears to be an irreversible inhibitor of mitochondrial respiration (Gardner et al., 1995; Xie and Wolin, 1996). The interaction of NO with O_2^- markedly enhances the potency of O_2^- as an inhibitor of respiration (Xie and Wolin, 1996). Since mitochondrial respiratory function is likely to influence signalling mechanisms involved in the metabolic control of blood flow that participate in microcirculatory P_{O_2}-elicited responses, conditions that promote O_2^- and $ONOO^-$ formation could influence blood flow responses linked to changes in P_{O_2}.

2.4. Potassium channels

There is substantial evidence that vascular potassium channels are regulated by signalling mechanisms elicited by changes in P_{O_2} (Weir and Archer, 1995). Several redox related systems have been linked to the control of potassium channel function. A well documented mechanism of opening calcium-regulated potassium channels in vascular tissue is through cGMP (Archer et al., 1994). Since the activity of sGC is controlled by multiple redox processes which were discussed previously, oxidant and NO derived mediators could control the opening of calcium-regulated potassium channels through changes in cGMP. There appears to be a cGMP-independent mechanism through which NO can open calcium-regulated potassium channels, and this process seems to involve modification of thiols (Bolotina et al., 1994). Thiol redox processes have also been

demonstrated to control the activity of potassium channels, such that oxidation appears to open the channels and reduction may be involved in their closing (Post et al., 1993; Weir and Archer, 1995). Thus, several P_{O_2} regulated redox processes have the potential to regulate the activity of potassium channels.

2.5. Evidence for the role of reactive O_2 species in O_2-elicited signalling mechanisms

Prostaglandins have been demonstrated to participate in P_{O_2}-elicited responses in several different vascular preparations (Kalsner, 1977; Roberts et al., 1981; Wolin et al., 1990; Omar et al., 1992). Studies in rat skeletal muscle arterioles (Wolin et al., 1990) and human placental arteries (Omar et al., 1992) have provided evidence that H_2O_2-elicited stimulation of relaxant and contractile prostaglandins, respectively, may participate in the responses of these vascular segments to post-hypoxic reoxygenation. In both of these preparations, the inhibition of prostaglandin biosynthesis results in the expression of a relaxation that appears to be mediated through the stimulation of sGC by H_2O_2 (Wolin et al., 1990; Omar et al., 1993a). The prostaglandin-mediated contraction to post-hypoxic reoxygenation and H_2O_2 is markedly enhanced in placental vessels derived from gestational diabetic patients associated with a loss of the relaxation response to exogenous and endogenous (lactate-derived) H_2O_2 (Figueroa et al., 1993, 1995). Thus, changes in P_{O_2} appear to elicit responses through stimulating prostaglandin production as a result of altering the levels of H_2O_2.

Studies in bovine pulmonary arteries provided evidence that P_{O_2} controls the levels of H_2O_2 metabolism by catalase and a cGMP-mediated relaxation, where hypoxia decreased both of these processes associated with the expression of a hypoxia-elicited contraction (Burke-Wolin and Wolin, 1989, 1990). In addition, an inhibitor of catalase (AT) was shown to attenuate this hypoxia-elicited contraction associated with an increase in force under normoxia (Burke-Wolin and Wolin, 1990). Studies on other vascular segments such as bovine coronary arteries have demonstrated (Mohazzab-H. et al., 1996a) that hypoxia decreases the levels of lucigenin-detectable O_2^- and H_2O_2 metabolism by catalase in a manner similar to bovine pulmonary arteries (Mohazzab-H. et al., 1995). However, the observed hypoxia-elicited relaxation of bovine coronary arteries appears to be mediated through a mechanism which does not involve O_2^--derived species or modulation of the activity of sGC. When endothelium-removed bovine coronary arteries are exposed to reoxygenation following hypoxia, these vessels show a transient relaxation that appears to be mediated through an increased production of O_2^--derived H_2O_2 and the stimulation of sGC (Mohazzab-H. et al., 1996a). As predicted by the mechanism shown in the model in Fig. 3, nitroblue tetrazolium has been observed to attenuate the contraction of bovine pulmonary arteries to hypoxia and the relaxation of bovine coronary arteries to post-hypoxic reoxygenation presumably through its action as a O_2^- scavenger which prevents the formation of H_2O_2. Tiron a scavenger of O_2^- which promotes H_2O_2 formation did not alter these P_{O_2}-elicited responses, further supporting the proposed role of P_{O_2} modulating the generation of H_2O_2 which is derived from endogenously produced O_2^- (Mohazzab-H. and Wolin, 1994; Mohazzab-H. et al., 1996a). As predicted by the model in Fig. 3, exposure of bovine pulmonary arteries to post-hypoxic reoxygenation causes a somewhat transient relaxation which is also attenuated by the inactivation of catalase with AT and by alcohols that prevent the stimulation of sGC by catalase-mediated H_2O_2 metabolism (Burke-Wolin and Wolin, 1989, 1990). Probes that inhibit GSH peroxidase (mercaptosuccinic acid (MS)) and that possess GSH peroxidase activity (ebselen) have helped confirm (Mohazzab-H. et al., 1999) in bovine coronary arteries the absence of a role for H_2O_2 in the relaxant response to hypoxia and the hypothesized role of H_2O_2 metabolism by catalase in relaxation caused by post-hypoxic reoxygenation (see Fig. 3). The relaxation to reoxygenation, but not the response to hypoxia, was enhanced by MS and attenuated by ebselen. In addition, inactivation of catalase by a pretreatment with AT essentially eliminated the relaxation to reoxygenation

in the absence or presence of MS, further supporting the proposed role of increased H_2O_2 metabolism by catalase in this response. In contrast the actions of these probes do not suggest a role for alterations in the redox status of GSH in the response to reoxygenation. These studies also provided evidence that the GSH peroxidase reaction has an important role in controlling the expression of responses linked to changes in H_2O_2 metabolism by catalase. Since diamide, an oxidant of glutathione with vasodilator activity (Archer et al., 1986), causes relaxation of bovine coronary arteries through a process that does not appear to involve the catalase-mediated stimulation of sGC (Mohazzab-H. et al., 1999), thiol oxidation may have additional mechanisms of promoting vascular relaxation. Thus, changes in P_{O_2} can elicit alterations in the function of endothelium-removed bovine coronary and pulmonary arteries through controlling the production of H_2O_2 and its metabolism by catalase, and this appears to control force generation through modulating the activity of sGC. Processes such as the activity of tissue GSH peroxidase and inhibition of catalase by substances including NO and O_2^- may prevent the expression of this P_{O_2} sensing mechanism, and this could permit other P_{O_2}-elicited signalling mechanisms to dominate the observed responses promoted by changes in P_{O_2}.

Recent studies have provided evidence for the important role of redox-associated changes in voltage-gated potassium channel function in pulmonary arterial P_{O_2}-elicited responses (Archer et al., 1993; Post et al., 1993; Weir and Archer, 1995). There is substantial evidence that redox processes control the activity of the potassium channels (Weir and Archer, 1995). For example, oxidized thiols appear to open potassium channels and cause hyperpolarization in pulmonary arterial smooth muscle cells. Whereas, reduced thiols seem to close these channels and the resulting depolarization is thought to cause the activation of voltage-gated calcium channels and vasoconstriction. In the redox model of potassium channel regulation by P_{O_2} in pulmonary arterial smooth muscle, it has been proposed by Archer et al. (1993) that hypoxia decreases the production of reactive O_2 species by sources including the mitochondrial electron transport chain and NAD(P)H oxidases. A key component of this hypothesized model is the impairment of mitochondrial function and NAD(P)H oxidases under

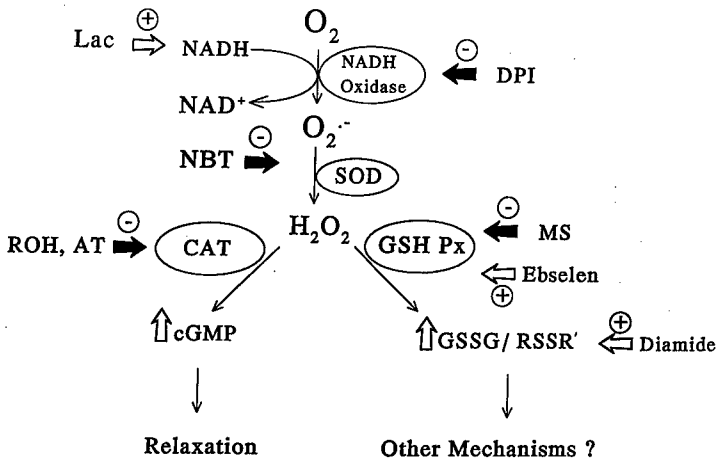

Fig. 3. Model for the potential role of NADH oxidase in the production of superoxide anion-derived H_2O_2 which appears to mediate in bovine coronary and pulmonary arteries P_{O_2} elicited responses potentially involved in the sensing of changes in P_{O_2} as a result of modulating of the activity of guanylate cyclase through alterations in peroxide metabolism by catalase (CAT). This mechanism and alternative signalling mechanisms potentially activated by changes in thiol redox are described in the text. Lac, lactate; DPI, diphenyliodonium; NBT, nitroblue tetrazolium; SOD, cytosolic or Cu, Zn-SOD; AT, 3-amino-1,2,4-triazole; ROH, alcohols including ethanol and methanol; GSH Px, glutathione peroxidase; MS, mercaptosuccinate; GSSG/RSSR', increased oxidized glutathione and *S*-thiolated proteins.

hypoxia also increases the level of reduced diffusible redox co-factors (such as NADH and GSH) in the cytosol. The increase in these co-factors leads to the observed decrease the opening of voltage-gated potassium channels as a result of an electron transfer process to a functional group on the channel. Thus, the P_{O_2} sensor in this model appears to be the components of mitochondria and NAD(P)H oxidase through which O_2 influences cytosolic redox. While the actual role of reactive O_2 species and redox processes in P_{O_2}-elicited vascular responses linked to the modulation of potassium channels remains to be established, it is also possible that cGMP may have a prominent role in the mediation of some of the observed effects of changes in P_{O_2} on the activity vascular potassium channels.

3. Potential roles for NAD(P)H oxidases in O_2 sensing

In the rat carotid body P_{O_2} appears to regulate the production of H_2O_2 and the redox status of a b-type cytochrome through a diphenyliodonium (DPI)-inhibitable flavoprotein. This led to the hypothesis that a phagocytic cell-type NAD(P)H oxidase could function as a P_{O_2} sensor protein (Cross et al., 1990). These observations resulted in the demonstration that a similar flavoprotein probe which inhibits the phagocytic-cell NADPH oxidase selectively attenuated the hypoxic vasoconstriction response in perfused rat lungs (Thomas et al., 1991). While studying the mechanism of relaxation to lactate in bovine pulmonary arteries, we recognized the potential importance of cytosolic NAD(H) redox in the control of O_2^- production and a relaxation that appeared to be mediated through H_2O_2 (Omar et al., 1993b). The presence of lactate was observed in these initial studies to increase the magnitude of the contraction to hypoxia. This observation is consistent with the source of H_2O_2 mediating the relaxation to lactate contributing to P_{O_2} sensing by this pulmonary vascular preparation. Subsequent studies characterizing the NADH oxidoreductase with NADH oxidase activity provided evidence for the presence of a phagocytic cell-like cytochrome b_{558} and that this oxidase appeared to show a P_{O_2} dependence for O_2^- generation which extended across the range of O_2 levels that promote vascular responses (Mohazzab-H. and Wolin, 1994). Endothelium also appears to possess an NADH oxidase activity that is regulated by cytosolic NAD(H) redox (Mohazzab-H. et al., 1994). The flavoprotein inhibitor DPI has also been observed to attenuate the contraction of bovine pulmonary arteries to hypoxia and the relaxation of bovine coronary arteries to post-hypoxic reoxygenation, associated with a decrease in O_2^- detection by intact and homogenized (NADH-dependent) preparations from these vascular segments (Mohazzab-H. et al., 1995, 1996a). Additional studies on the vascular NADH oxidase revealed the presence and essential role of a p22phox subunit which is extremely similar to the p22phox subunit of the phagocytic NADPH oxidase system (Griendling and Ushio-Fukai, 1997). While the phagocytic oxidase contains a gp91phox subunit, the presence of a subunit of this type in the vascular oxidase has been more difficult to establish. There seems to be a lack of evidence for the presence of the mRNA for this subunit in vascular smooth muscle (Griendling and Ushio-Fukai, 1997). However, antibodies to the gp91phox of the phagocytic oxidase have detected similar proteins in vascular smooth muscle (Marshall et al., 1996) and the response of pulmonary arteries to hypoxia is not markedly altered in a knock-out mouse model for the phagocytic NADPH oxidase (Weir et al., 1996). These observations suggest the presence in vascular smooth muscle of a NADH oxidase subunit which resembles the gp91phox subunit of the phagocytic NADPH oxidase. Overall, vascular smooth muscle appears to possess a flavoprotein containing NADH oxidase which shows a P_{O_2} dependent production of superoxide anion that is consistent with it functioning as a key O_2 sensor in vascular smooth muscle.

4. Implications the potential roles of NAD(P)H oxidases and reactive O_2 species in the regulation of vascular function by P_{O_2}

Several observations support a potentially important role for the function of a NAD(P)H oxidases as a P_{O_2} sensor in vascular tissue which stimulates responses through the generation of O_2^--derived metabolites, such as H_2O_2. In the perfused rabbit lung, the flavoprotein probe DPI, which inhibits NAD(P)H oxidases causes a selective attenuation of hypoxic vasoconstriction associated with an increase in pulmonary artery pressure under normoxia (Grimminger et al., 1995). In addition, nitroblue tetrazolium, a O_2^- scavenger which prevents H_2O_2 formation, was also observed in the rabbit lung preparation to selectively attenuate the hypoxic pulmonary vasoconstriction response (Weissmann et al., 1998). Studies on perfused rat lungs and isolated bovine and cat pulmonary arteries have demonstrated that the DPI-like inhibitors attenuate the contraction to hypoxia (Thomas et al., 1991; Mohazzab-H. et al., 1995; Marshall et al., 1996). However, the DPI-like inhibitors do not increase force generation under normoxia, suggesting the possibility of a mechanism other than the removal of a H_2O_2 relaxation in the response to hypoxia or that the DPI-type probes may have additional actions on force generation by pulmonary arteries. For example, DPI-type probes have been shown to inhibit the function of multiple flavoproteins (O'Donnell et al., 1994) and to inhibit potassium and calcium currents in pulmonary arterial smooth muscle (Weir et al., 1994). In saline perfused rat lungs in which NO synthase was blocked, pretreatment with the catalase inhibitor AT causes an increase in basal perfusion pressure associated with a decrease in hypoxic vasoconstriction suggesting the potentially important role of H_2O_2 metabolism by catalase in this response (Monaco and Burke-Wolin, 1995). Rat skeletal muscle arterioles show an in vivo post-hypoxic reoxygenation response (reactive hyperemia) which seems to utilize mechanisms involving prostaglandins and the stimulation of sGC that are similar to the vasodilator response to H_2O_2 in this vascular preparation (Wolin et al., 1990). Interestingly, rat skeletal muscle arterioles also possess a vasodilator response to lactate that is attenuated by the flavoprotein probe DPI and antagonists of the stimulation of sGC (Chen et al., 1996), suggesting the presence of a functionally important NADH oxidase in this preparation. All of these observations suggest the potentially important role of NADH oxidase as a P_{O_2} sensor in vascular tissue and H_2O_2 as a key mediator which activates signalling mechanisms that control force generation in certain P_{O_2}-elicited vascular responses. Observations on the ways through which components of the signalling mechanisms mentioned in this review function hint at the potential versatility of the processes involved and the ways these systems could be altered in applied physiological and pathophysiological states. While any source of production of reactive O_2 species that is controlled by changes in physiological levels of O_2 could function as an O_2 sensor, observations described in this review suggest the fundamental importance of a NADH oxidase linked to the redox status of cytosolic NAD(H) as a P_{O_2} sensor in vascular tissue. An increase in the activity of other sources of production of reactive O_2 and NO derived species in vascular pathophysiological states by systems including (Wolin, 1996) NO synthase, the phagocytic cell-type NADPH oxidase, cytochrome P450, mitochondria and xanthine oxidase may change the oxidase that functions as the primary P_{O_2} sensor and signalling mechanisms that mediate O_2 regulated responses resulting in the expression of alterations in the control of blood flow by changes in P_{O_2} and metabolism.

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Oxygen sensing and signaling: impact on the regulation of physiologically important genes

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Abstract

A growing number of physiologically relevant genes are regulated in response to changes in intracellular oxygen tension. It is likely that cells from a wide variety of tissues share a common mechanism of oxygen sensing and signal transduction leading to the activation of the transcription factor hypoxia-inducible factor 1 (HIF-1). Besides hypoxia, transition metals (Co^{2+}, Ni^{2+} and Mn^{2+}) and iron chelation also promote activation of HIF-1. Induction of HIF-1 by hypoxia is blocked by the heme ligands carbon monoxide and nitric oxide. There is growing, albeit indirect, evidence that the oxygen sensor is a flavoheme protein and that the signal transduction pathway involves changes in the level of intracellular reactive oxygen intermediates. The activation of HIF-1 by hypoxia depends upon signaling-dependent rescue of its α-subunit from oxygen-dependent degradation in the proteasome, allowing it to form a heterodimer with HIF-1β (ARNT), which then translocates to the nucleus and impacts on the transcription of genes whose cis-acting elements contain cognate hypoxia response elements.

Keywords: Gene regulation, oxygen sensing; Hypoxia, hypoxia-inducible factor 1, cellular sensor, signal transduction; Oxygen, sensing, genes

1. Introduction

Man and other mammals adapt to hypoxia by a number of physiologically appropriate responses, such as increased production of erythropoietin (Epo) which augments the red cell mass, induction of tyrosine hydroxylase (TH) which facilitates the control of ventilation via the carotid body, and stimulation of new blood vessels by up-regulation of vascular endothelial growth factor (VEGF) (Bunn and Poyton, 1996). The regulation of the genes encoding these proteins depends upon accurate sensing of p_{O_2} and transduction of a signal that activates HIF-1, a heterodimeric transcrip-

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tion factor that enables enhanced transcription (Guillemin and Krasnow, 1997). It is likely that the mechanism for the sensing of oxygen and its subsequent signaling departs from well established systems of receptor binding. Due to oxygen's peculiar chemical properties, there is a limited repertoire of molecules to which it can combine. Oxygen is known to bind to and react with heme proteins (and, in certain invertebrates, hemerythrin, hemocyanin and chlorocruorin). Heme proteins play a critical role in oxygen sensing by bacteria and yeast (Bunn and Poyton, 1996). Considering that higher eukaryotes are exposed to less varied environmental stimuli but must respond to more diverse and complex internal stimuli, it is likely that they have become endowed with more elaborate and delicate mechanisms for oxygen sensing and signal transduction. In all vertebrates and many non-vertebrates, oxygen transport depends on the circulation of erythrocytes which enable oxygen unloading to tissues at relatively high O_2 tension. Therefore in order to monitor perturbations in oxygen transport there is need for sensors with relatively low oxygen affinity.

Initial work on oxygen sensing and signal transduction began with studies on neural transmission in the carotid body and erythropoietin production in hepatic cell lines. The subsequent discovery of HIF-1 (Wang et al., 1995) and the realization that the sensing and signaling process is probably shared among many if not all types of cells (Maxwell et al., 1993) have prompted experiments in a variety of other experimental systems. HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β (ARNT) subunits, both of which belong to the basic helix–loop–helix PAS family (Wang and Semenza, 1993b; Wang et al., 1995). At the mRNA level, both HIF-1α and ARNT genes are constitutively expressed and not significantly up-regulated by hypoxia (Gradin et al., 1996; Huang et al., 1996; Wood et al., 1996; Kallio et al., 1997). Whereas changes in oxygen tension fails to affect ARNT protein abundance, hypoxia markedly increases levels of HIF-1α (Wang et al., 1995; Huang et al., 1996; Kallio et al., 1997), rescuing the subunit from oxygen-dependent degradation in the proteasome (Huang et al., 1998). Thus, hypoxia-induced activation of HIF-1 depends in part on post-translational stabilization of HIF-1α.

1.1. Carotid body

Ventilation in mammals, birds and perhaps fish, is regulated in part by the carotid body (Dejours, 1981; Schmidt-Nielson, 1990). This very small and highly vascular organ, located in man at the bifurcation of the carotid artery, is composed of glomus type I chemoreceptor cells which, upon challenge with hypoxia, release neurotransmitters that set the level of electrical activity in the afferent fibers of the carotid sinus nerve. Type I cells have voltage dependent K^+, Na^+ and Ca^{++} channels (López-Barneo et al., 1988). Patch clamp studies on isolated plasma membranes from type I cells have shown that a single K^+ channel type is down-regulated by lowering oxygen tension (Ganfornina and López-Barneo, 1991). The rapid response indicates that the oxygen sensing mechanism is independent of transcription and translation. Moreover, this physiologically relevant oxygen sensor appears to be localized in the plasma membrane as opposed to somewhere in the cell interior. The addition of relatively low levels of carbon monoxide to the hypoxic gas mixture substantially reverses the inhibition of K^+ currents (López-López and González, 1992) and the chemosensory nerve discharge (Lahiri et al., 1993). In excitable O_2 sensitive cells, intracellular free Ca^{2+} may be a primary mechanism for gene regulation (Raymond and Millhorn, 1997).

1.2. Erythropoietin production

Erythropoietin is a glycoprotein hormone that regulates proliferation and differentiation of erythroid cells (Jelkmann, 1992; Porter and Goldberg, 1993). A large number of classic physiologic studies demonstrated that Epo production is markedly enhanced by hypoxia. The only other known stimulus to Epo production in vivo is the administration of certain transition metals. Experiments with intact animals (Goldwasser et al., 1958) as well as perfused kidneys (Fisher and Langston, 1968) demonstrated that cobaltous chloride stimulates erythropoiesis by increasing

the production of Epo. Intrarenal injections of nickel have also been shown to induce erythrocytosis (Jasmin and Solymoss, 1975; Morse et al., 1977). When human hepatoma cells (Hep3B or HepG2) were incubated for 24 h in the presence of increasing amounts of $CoCl_2$ or $NiCl_2$ there was a dose-dependent enhancement of Epo mRNA expression (Goldberg et al., 1988; Fandrey and Bunn, 1993) and protein production (Goldberg et al., 1988) similar to that observed with increasing degrees of hypoxia. When other transition metals (manganese, zinc, iron, cadmium, and tin) were tested, only manganese induced measurable Epo protein, but less than that obtained by either cobalt or nickel. Hypoxic induction of erythropoietin protein (Goldberg et al., 1988) and mRNA (Huang et al., 1999) in Hep3B cells was markedly inhibited by the presence of carbon monoxide (CO). In contrast to its effect in hypoxic cells, CO did not inhibit the induction by cobalt or nickel of Epo protein (Goldberg et al., 1988) or mRNA (Huang et al., 1999).

2. Evidence that the sensor is a heme protein

The effect of carbon monoxide on oxygen sensing signaling extends beyond the carotid body and the production of erythropoietin in Hep3B cells. CO has also been shown to offset hypoxia's effect on the expression of VEGF (Goldberg and Schneider, 1994), platelet derived growth factor (Morita and Kourembanas, 1995), endothelin-1 (Morita and Kourembanas, 1995) and phosphoenolpyruvate carboxykinase (Kietzmann et al., 1993). More recently, Liu et al. (1998) and our laboratory (Huang et al., 1999) have shown that CO suppresses the hypoxic activation of HIF-1. Carbon monoxide has remarkable specificity in biological systems, binding non-covalently to ferrous heme groups in hemoglobin, myoglobin, certain cytochromes and other heme proteins. Thus these experiments strongly support the hypothesis that the oxygen sensor is a heme protein. The results with CO indicate that the affinity of this ligand for the heme group in the sensor is low, compared to hemoglobin which binds CO over 200-fold more tightly than oxygen. It is likely that the sensor's low affinity for CO has adaptive significance. The primary toxicity of CO in higher organisms is due to its high affinity binding to hemoglobin, locking the tetramer in the 'oxy' comformation and thereby increasing oxygen affinity and decreasing oxygen unloading to tissues. When subjected to CO-induced hypoxic stress, the organism needs intact oxygen sensors. These sensors would be unresponsive if they had high affinity for CO.

The induction of Epo by the transition metals Co^{2+}, Ni^{2+} and Mn^{2+} led to experiments which support the hypothesis that cobalt, nickel and manganese atoms substitute for the iron atom in the heme moiety of the oxygen sensor (Goldberg et al., 1988). Cobalt has been shown to be a substrate for ferrochelatase, the enzyme responsible for the incorporation of iron into protoporphyrin IX to make heme (Labbe and Hubbard, 1961). Radiolabeling studies both in vivo (Sinclair et al., 1979) and in cultured cells (Sinclair et al., 1982) have demonstrated the incorporation of transition metals including cobalt and nickel into heme. If the effect of cobalt and nickel depends on incorporation into the heme moiety, increased levels of iron should competitively inhibit the stimulatory effects of these metals on Epo gene expression. We have provided experimental support for this prediction (Ho and Bunn, 1996). The response to cobalt is not specific for the Epo gene or for Epo producing cells. For example, cobalt has been shown to mimic hypoxia in stimulating the expression of VEGF (Goldberg and Schneider, 1994; Ladoux and Frelin, 1994; Minchenko et al., 1994) and glycolytic enzymes (Firth et al., 1994; Semenza et al., 1994; Ebert et al., 1996) in a number of different types of cells Moreover, reporter gene experiments show that Epo's 3′ enhancer (discussed in detail below) is responsive to both hypoxia and cobalt in a variety of cell lines from different tissues (Maxwell et al., 1993). The fact that CO does not block the induction of Epo by cobalt or nickel (Goldberg et al., 1988; Huang et al., 1999) is consistent with the inability of cobalt and nickel substituted hemes to bind to CO.

Since nitric oxide (NO), like CO is a gaseous ligand that binds to ferrous atoms in heme proteins, it is of interest to determine its impact

on oxygen sensing and signaling. Several laboratories have shown that NO from nitroprusside or other donors suppresses hypoxic activation of HIF-1 DNA binding, the stabililzation of HIF-1α protein and the induction of reporter genes containing HIF-1 response elements (Liu et al., 1998; Sogawa et al., 1998; Huang et al., 1999).

The best studied system of oxygen sensing and signal transduction has been in nitrogen-fixing bacteria, Rhizobium, whose oxygen sensor has been shown to be an oxygen-binding heme protein containing a protein kinase domain (Gilles-Gonzalez et al., 1991, 1994; Rodgers et al., 1996; Lukat-Rodgers and Rodgers, 1997).

The evidence summarized above for a central role of heme protein(s) has led to the proposal of two clearly distinct sites and mechanisms for oxygen sensing and signaling:[1] (a) a cytochrome *b*-like NAD(P)H oxidase on the plasma membrane; and (b) mitochondrial complex IV cytochrome oxidase

3. Cytochrome b-like NAD(P)H oxidase

3.1. Role of peroxide in signaling

Fandrey et al. (1994) showed that the induction of Epo production, following exposure of Hep3B cells to hypoxia, could be aborted by the addition of either hydrogen peroxide, menadione, an agent which increases intracellular peroxide production, or aminotriazole, an inhibitor of catalase. As mentioned above, treatment of Hep3B cells with cobalt induces Epo production. HepG2 cells exposed to cobalt undergo a significant decrease in production of hydrogen peroxide (Görlach et al., 1994). Taken together, as shown in Fig. 1, these experiments suggest that molecular oxygen is chemically reduced, presumably by the sensing apparatus, to superoxide and peroxide, thereby providing a plausible chemical signal that could impact on the activity of HIF-1, which in turn regulates oxygen responsive genes.

In cells containing hydrogen peroxide, highly reactive oxygen compounds such as hydroxyl radical and singlet oxygen can be formed. The generation of these reactive oxygen species (ROS) is catalyzed by free iron via the Fenton reaction. Virtually all genes that are inducible by hypoxia are also up-regulated by desferrioxamine and other strong chelators of iron (Wang and Semenza, 1993a; Semenza et al., 1994; Gleadle et al., 1995a). As depicted in Fig. 1, it is likely that drastic reduction in intracellular free iron lowers the level of ROS, thereby mimicking a hypoxic environment.

3.2. Spectral analyses

Support for the central role of an oxidase has been provided by Acker (1994a,b) who have made direct spectral measurements of cells in which oxygen sensing plays a critical role. They have obtained difference spectra in the visible range in the presence and absence of inhibitors of respiratory cytochromes in both Type I carotid body cells (Cross et al., 1990) and in HepG2 cells (Görlach et al., 1993). In each case, their spectral data could be deconvoluted to suggest the presence of a *b*-like cytochrome. This heme protein was estimated to comprise ~6% of the total cytochrome b in the cell. It appeared to bind oxygen with relatively low affinity and also carbon monoxide (Görlach et al., 1993). Moreover, cobalt treatment of HepG2 cells abolished the response of this b-like cytochrome to hypoxia whereas the redox states of mitochondrial cytochromes c and aa3 were unaffected (Görlach et al., 1994). Although these absorbance measurements are potentially of considerable importance and value, they are inherently difficult to execute and to interpret, owing to a low ratio of signal to noise.

3.3. Tissue localization

The spectral evidence that the oxygen sensor is a *b* cytochrome led Acker et al. to focus on

[1] Recently, Srinivas et al. (1998) have proposed yet another model of oxygen sensing. They expressed the α-subunit of the heterodimeric transcription HIF-1 as a GST-fusion protein in E. coli and reported that it contains up to 2 moles of non-heme iron per mole protein. They suggested that the iron atoms on this protein could bind O_2, thereby serving as a non-heme sensing mechanism that would impact directly on HIF-1 activation. However, the authors have subsequently retracted this report [J. Biol. Chem. 74, 1180].

neutrophil-macrophage cytochrome b_{558}, which has similar spectral properties and, importantly, functions as a NAD(P)H oxidase, converting oxygen to superoxide, in keeping with the postulated role of reactive oxygen compounds in signaling. They demonstrated by Western blot analysis that both type 1 cells of the carotid body (Kummer and Acker, 1995) and HepG2 cells (Görlach et al., 1993) contain at least two of the subunits ($p22_{phox}$ and $p47_{phox}$) of the oxidase. However, the specific subunits that compose the NADPH oxidase in neutrophils and macrophages are unlikely to play important roles in oxygen sensing since patients with genetic subtypes of chronic granulomatous

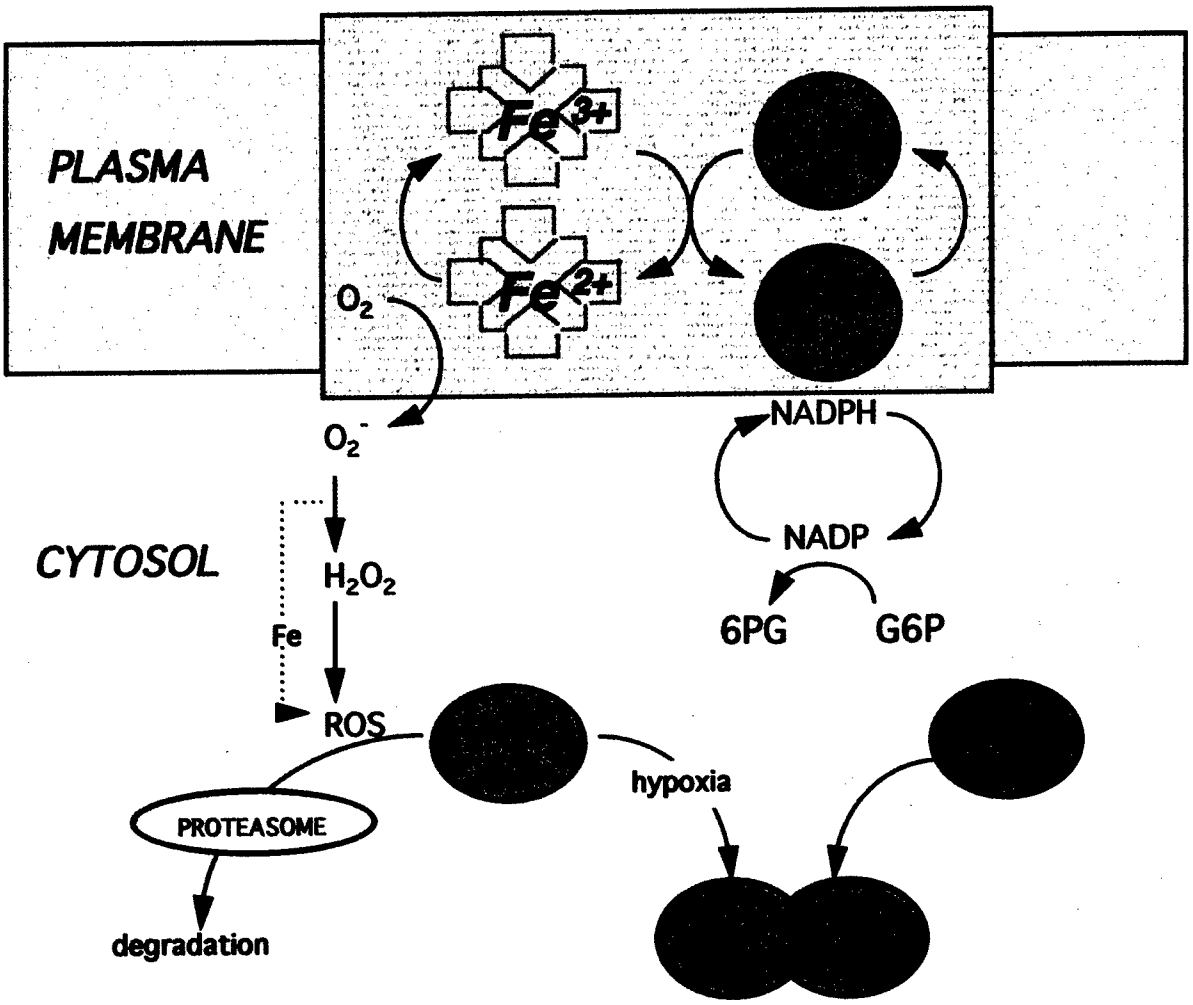

Fig. 1. Proposed model of oxygen sensing and signaling. In oxygenated cells, a flavo-heme protein in the plasma membrane functions as an NADPH oxidase, transferring electrons through the flavin and heme to molecular oxygen, generating superoxide O_2^- which in the presence of iron is converted to $OH^\cdot$ and other reactive oxygen species. As a result HIF-1α is oxidatively modified so that it is recognized by the proteasome and rapidly degraded. At low oxygen tension, HIF-1α is stable and can form a heterodimer with constitutively expressed HIF-1β, thereby activating HIF-1 which translocates to the nucleus and binds to response elements in hypoxia inducible genes.

disease, characterized by absence or abnormality of these subunits, have a very restricted clinical phenotype with no apparent evidence of disordered oxygen sensing. Moreover, lymphoid cell lines from patients deficient in $gp91_{phox}$, $p22_{phox}$, when transfected with an oxygen sensitive reporter genes, showed normal responses to hypoxia (Wenger et al., 1996).

Additional support for the role of an NAD(P)H oxidase in oxygen sensing has come from experiments utilizing iodonium compounds (Goldwasser et al., 1995; Gleadle et al., 1995b) which inhibit this type of enzyme along with other flavoproteins (Stuehr et al., 1991). Diphenylene iodonium (DPI) transiently increased the spontaneous neural discharge in isolated perfused carotid body preparations and blocked the hypoxia-induced increase in discharge (Cross et al., 1990). The same result was observed with pulmonary neuroepithelial cells (Youngson et al., 1993). However, interpretation of these results is confounded by the observation that DPI is a non-specific inhibitor of ion channels (Wyatt et al., 1994).

Despite major gaps in our understanding, it seems likely that most cells share a common oxygen sensing apparatus which is depicted schematically in Fig. 1. The sensor is likely to be a cytosolic, membrane bound, multisubunit b-like cytochrome which binds oxygen and reduces it to superoxide, thereby generating ROS which serve as chemical signals that impact on the transcription factor HIF-1 that regulates oxygen responsive genes. This general model accommodates a considerable body of physiologic, biochemical and genetic evidence described above. In its simplest form this scheme would provide a continuous monitor of intracellular oxygen tension over a wide range.

4. Mitochondrial complex IV heme protein

Since the mitochondrion is the primary site of oxygen metabolism, this organelle might seem to be a logical site for sensing and initiation of signal transduction. As mentioned above, patch clamp experiments indicate that the oxygen sensor in carotid body type I cells resides in the plasma membrane (Ganfornina and López-Barneo, 1991). Nevertheless, several reports have suggested that mitochondria may play a critical role in oxygen sensing by the carotid body (Mulligan et al., 1981; Obeso et al., 1985; Duchen and Biscoe, 1992; Wilson et al., 1994; Lahiri et al., 1995). In view of the extraordinarily rich vascularity and high oxygen consumption of the carotid body, there may be a unique functional role for a mitochondrial oxygen sensor.

Recently, Schumacker et al. (Chandel et al., 1997, 1999; Duranteau et al., 1998) have presented evidence that mitochondrial cytochrome oxidase (Complex IV) serves as an oxygen sensor in other cell types: hepatocytes and cardiac myocytes. These investigators observed that when cells are exposed to moderate degrees of hypoxia (20 torr = 3% O_2), oxygen uptake decreased significantly, owing to a significant reduction in the Vmax of cytochrome oxidase. This effect was noted after a latency of ~ 2 h and was fully reversible. Measurements of mitochondrial membrane potential indicated that the decrease in respiration following hypoxia was due solely to a direct inhibition of mitochondrial proton pumping and not to decreased ATP utilization. Mitochondria are a major source of superoxide (O_2^-) owing to inefficient transfer of electrons in the respiratory chain. Accordingly, Schumacker's group performed a set of experiments to test whether superoxide produced proximal to mitochondrial Complex III serves as a signalling molecule. They showed that graded decreases in p_{O_2} from 35 to 7 torr (5–1% O_2) resulted in a progressive increase in ROS, as measured by the fluorescent probe dichloro-fluoriscein. The addition of inhibitors supported the mitochondria as a source of the induced ROS. Rotenone and TTFA (inhibitors of Complexes I and II, respectively) suppressed formation of ROS, whereas antimycin A and azide (inhibitors of Complex III and IV, respectively) caused an increase in ROS. These investigators then showed that HIF-1 activation and Epo gene expression in Hep3B cells correlated directly with changes in ROS, induced either by mitochondrial inhibitors or by reducing agents (Chandel et al., 1999). Moreover, in ρ° Hep3B cells, whose mitochondria have been destroyed by

ethidium bromide, hypoxia failed to induce either HIF-1 activity or Epo gene expression. In contrast, when ρ° Hep3B cells are exposed to cobalt, HIF-1 is activated and Epo mRNA is up-regulated. The model for oxygen sensing suggested by these findings is in direct conflict with the one described above, in which a cytochrome b-like oxidase generates decreased levels of ROS during hypoxia.

Considerably more experimental work is needed in order to establish whether either this mitochondrial model or the NADPH oxidase model of oxygen sensing and signaling is correct.

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Regulation of gene expression and secretory functions in oxygen-sensing pheochromocytoma cells

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Abstract

The cellular response to hypoxia is complex. Specialized oxygen chemosensitive cells that are excitable respond to reduced O_2 by membrane depolarization, altered gene expression, and neurotransmitter secretion. We have used the O_2-sensitive pheochromocytoma (PC12) cell line to investigate the cellular response to hypoxia. Here, we present evidence that membrane depolarization and increased intracellular free Ca^{2+} are major regulatory events in these cells. Membrane depolarization is mediated by the inhibition of a slow-inactivating voltage-dependent potassium (K) channel. Evidence from molecular biology and patch-clamp studies indicate that the O_2-sensitive K channel is a member of the Kv1 family. We also reviewed findings on the regulation of gene expression in PC12 cells during hypoxia. An increase in intracellular free Ca^{2+} is required for hypoxia-induced transcription of a number of genes including tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of catecholamine neurotransmitters, and several of the immediate early genes. We also reviewed the role of dopamine (DA) and adenosine (ADO) receptors in regulation of membrane depolarization and gene expression.

Keywords: Channels, K^+, oxygen-sensitive; Gene regulation, hypoxia; Hypoxia, cellular response, PC12; Pheochromocytoma cells, hypoxic response; Receptors, adenosine, dopamine

1. Introduction

Survival of mammalian cells depends on the availability of oxygen. Specialized chemosensitive cells in the body are responsible for maintaining the appropriate O_2 concentration in the blood. These cells can be found in a variety of tissues including the carotid body (an organ located at the bifurcation of the common carotid artery), the pulmonary vasculature, and pulmonary neuroepithilial bodies (small organs distributed widely throughout the airway mucosa). Exposure of

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these O_2-sensitive cells to hypoxia results in an array of central, cardiovascular and pulmonary responses that optimize the delivery of O_2 to vital organs, thereby preventing global or localized O_2 deficits that can produce irreversible cellular damage (Weir and Archer, 1995; Lahiri, 1997). Despite their important homeostatic role, the mechanisms by which O_2-sensitive cells detect a change in O_2 tension (P_{O_2}) and transduce this signal into an appropriate physiological response are still poorly understood. New insights concerning the mechanisms of O_2 sensitivity have been obtained in experiments using an O_2-sensitive clonal cell line: the pheochromocytoma (PC12) cell line. PC12 cells, the neoplastic counterpart of adrenal chromaffin cells (Green and Tischler, 1987), are excitable exocrine cells that are highly sensitive to changes in O_2 availability (Zhu et al., 1996). These cells are propagated readily in culture and are characterized by numerous secretory granules. They are similar to type I carotid body cells (the O_2-sensing cells of the carotid body) with respect to embryonic origin (neural crest), morphology, and their dopaminergic phenotype (Green and Tischler, 1987). Like other oxygen chemosensitive cells, PC12 cells respond to acute exposure to hypoxia with immediate responses such as membrane depolarization, an increase in intracellular Ca^{2+}, and release of neurotransmitters (Zhu et al., 1997; Kobayashi et al., 1998; Kumar et al., 1998; Taylor and Peers, 1998). Prolonged exposure of PC12 cells to hypoxia results in changes in O_2-sensitivity and expression of specific genes and their cognate proteins which are known to play a role in the cellular response to hypoxia (Czyzyk-Krzeska et al., 1994; Conforti and Millhorn, 1997). Similar changes in gene expression have been observed in the carotid body type I and other O_2-sensitive cells (Czyzyk-Krzeska et al., 1992; Bunn and Poyton, 1996). Thus, the physiological response to reduced O_2 tension in PC12 cells is similar to the response in excitable O_2-sensitive tissues. Hence, PC12 cells provide a unique and useful model in studying the molecular mechanisms underlying O_2 chemosensitivity, as they don't have the same limitations as chemosensitive tissues (i.e. small numbers of O_2-sensitive cells present and cell heterogeneity).

The current review focuses on the different responses elicited by hypoxia in PC12 cells (summarized in Fig. 1) and the underlying mechanisms. A change in environmental P_{O_2} constitutes a signaling stimulus that affects ion channel activity, Ca^{2+} homeostasis and regulation of genes involved in transmitter synthesis. A primary response to hypoxia in PC12 cells is facilitation of release of neurotransmitters such as dopamine (DA), norepinephrine (NE) and adenosine (ADO). Although the role of these transmitters in transduction of the hypoxic stimulus is still controversial, recent findings show that DA and ADO exert a feedback control on cellular excitability and function in PC12 cells during hypoxia via stimulation of D_2 and A_2 receptors, respectively (Zhu et al., 1997; Kobayashi et al., 1998).

2. Ion channel activity

PC12 cells express an O_2-sensitive K^+ (K_{O_2}) current that is inhibited by hypoxia. Inhibition of this current induces membrane depolarization, an

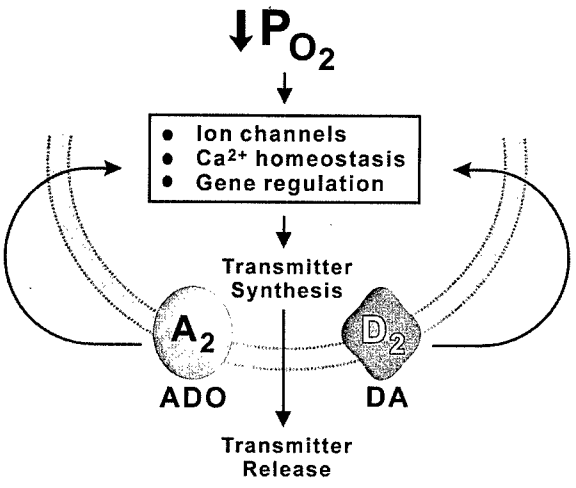


Fig. 1. Schematic of the effect of a decrease in O_2 tension (P_{O_2}) in the environment on PC12 cellular function. Exposure to low P_{O_2} (hypoxia) affects ion channel activity, intracellular free Ca^{2+} concentration and gene expression and ultimately promotes transmitter synthesis and release. Of the known transmitters released during hypoxia, DA and ADO can feedback to regulate cell excitability. This feedback regulation occurs via stimulation of the D_2 and A_2 receptors present in PC12 cells.

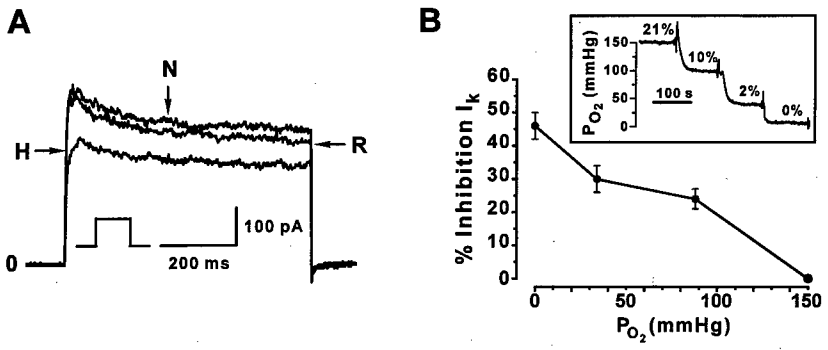


Fig. 2. Oxygen-sensitive K^+ current in PC12 cells: (A) Whole-cell K^+ current were elicited by depolarizing voltage steps from −90 mV HP to +50 mV. Currents were recorded during control period in normoxia (N, P_{O_2} = 150 mmHg), after steady-state inhibition by hypoxia (H, P_{O_2} < 10 mmHg) and after returning to normoxic conditions (R); (B) Percentage inhibition of K^+ current at different P_{O_2}. Cells were exposed to solutions with progressively lower P_{O_2} (n = 6, same step potential as in A). Current inhibition is reported as relative changes in current amplitude from the normoxia values. Inset: P_{O_2} in the recording medium as monitored continuously with an O_2 electrode in the chamber.

increase in intracellular Ca^{2+} concentration, gene regulation and, ultimately, transmitter release (Czyzyk-Krzeska et al., 1994; Zhu et al., 1996; Raymond and Millhorn, 1997). The inhibitory effect of hypoxia on the K_{O_2} current is shown in Fig. 2. Outward slow-inactivating voltage-dependent K^+ currents are elicited in PC12 cells by depolarizing voltage steps (Fig. 2A). Exposure to hypoxia (H, P_{O_2} < 10 mmHg) induces inhibition of the outward K^+ current and this effect is reversible upon returning to normoxia (N, 150 mmHg). The magnitude of hypoxia-induced inhibition of the K^+ current depends on the severity of hypoxia. Perfusion with progressively lower P_{O_2} reduces the K^+ current in a stepwise fashion, as shown in Fig. 2B. Therefore, an outward K^+ current that is selectively and reversibly inhibited by hypoxia is present in PC12 cells. This K_{O_2} current is a slow-inactivating voltage-dependent K^+ current, which is blocked by tetraethylammonium (TEA), a blocker of voltage-dependent K^+ (K_v) channels. Fig. 3A illustrates the characteristics of the K_{O_2} current by showing the relative inhibition of the K^+ current induced by hypoxia under various experimental conditions. In control conditions, hypoxia inhibits the K^+ current by ~20%. Exposure of cells to 5 mM external TEA results in loss of the hypoxia-induced inhibition of the K^+ current. Moreover, the K_{O_2} current is not sensitive to Ca^{2+} or to the holding voltage. In fact, the same percentage inhibition of the K^+ current by hypoxia was observed in experiments performed in Ca^{2+}-free medium and in experiments where the holding potential was kept at −30 mV. Therefore the K_{O_2} current in PC12 cells is neither a Ca^{2+}-activated K^+ current nor a transient K^+ current. These currents are in fact carried by voltage-dependent K^+ (K_v) channels whose activity is modulated by Ca^{2+} or holding potential, respectively.

O_2-sensitive K^+ currents have been characterized in other chemosensitive cells. In most chemosensitive cells such as carotid body type I cells and rat pulmonary artery smooth muscle cells the K_{O_2} current is voltage-dependent; however, in rat carotid body, a background K^+ current was also recently shown to be inhibited by hypoxia (Peers, 1990; Ganfornina and López-Barneo, 1992; Archer et al., 1996; Buckler, 1997). Single channel experiments have shown that the K_{O_2} current is carried by specific O_2-sensitive voltage-dependent K^+ channels. In rabbit carotid body type I cells three types of K^+ channels have been described: a 20 pS O_2-sensitive Kv channel, a large-conductance Ca-activated K^+ (K_{Ca}) channel, and a small-conductance Kv channel (Ganfornina and López-Barneo, 1992). In the pulmonary artery a 37 pS delayed rectifier O_2-sensitive K^+ (K_{dr}) and the K_{Ca} channel are present (Archer et al., 1996). Four types of K^+ channels were recorded in PC12 cells: a small conductance K^+ channel (14 pS), a cal-

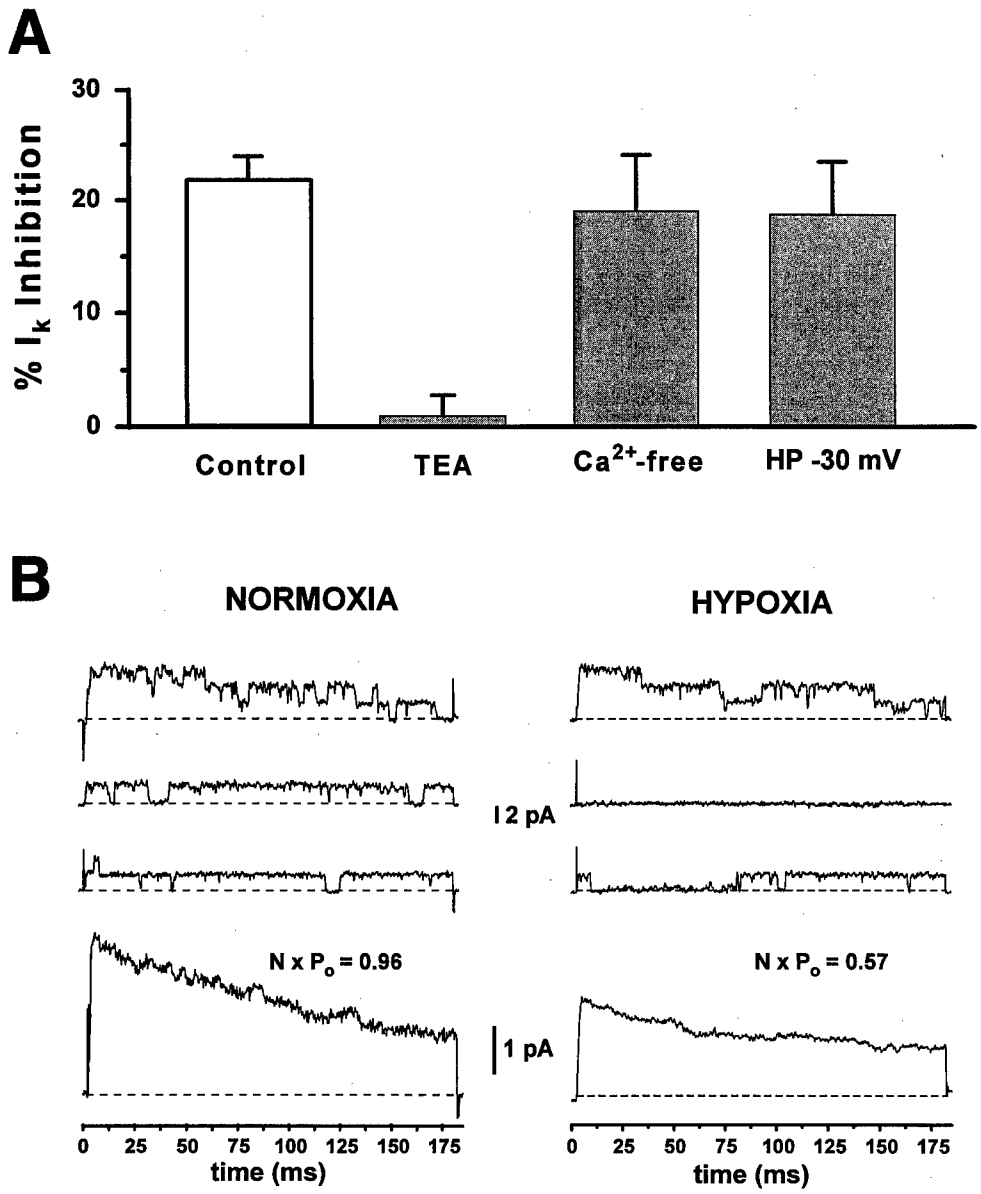


Fig. 3. (A) Properties of the O_2-sensitive K^+ current in PC12 cells. K^+ currents were recorded in whole-cell configuration at +50 mV for 800 ms, from −90 mV HP (or otherwise indicated). Current inhibition by hypoxia (0 mmHg) was measured in different experimental conditions: under control conditions (open bar, HP = −90 mV, standard bath solution with 2 mM $CaCl_2$ and Ca^{2+}-free pipette solution, $n = 11$), in the presence of 5 mM TEA ($n = 4$), in Ca^{2+}-free external medium ($n = 5$) and using an HP of −30 mV ($n = 5$); (B) Characterization of the K_{O_2} channel. Top panels: representative traces recorded in a cell-attached patch during step depolarizing pulses (from −60 to +50 mV, 180 ms duration) in an asymmetrical K^+ gradient: 140 mM external K^+ and 2.8 mM K^+ in the pipette. Currents were recorded in normoxia (21% O_2) and 2 min after exposure to hypoxia (10% O_2). Leak and capacitative currents were subtracted from the record. Upward current deflections from the zero line correspond to the opening of the channel. The corresponding ensemble-averaged currents (from 100 consecutive traces) are shown in the bottom panels. Dashed lines represent the zero current. Values for channel activity are given as $N \times P_O$, where N corresponds to the number of functional channels in the patch and P_O is the open probability.

cium-activated K^+ (K_{Ca}) channel (102 pS) and two K^+ channels with similar conductance (20 pS; Conforti and Millhorn, 1997; Hoshi and Aldrich, 1988). These last two channels differ in their time-dependent inactivation: one is a slow-inactivating channel, while the other belongs to the family of fast transient K^+ channels. Hypoxia selectively inhibits only the 20 pS slow-inactivating delayed-rectifier type of K^+ channel in PC12 cells (Conforti and Millhorn, 1997). Fig. 3B shows the single-channel properties of the 20 pS K_{O_2} channel recorded in a cell-attached patch in PC12 cells and the effect of hypoxia on the channel activity. Single-channels currents were elicited by step-pulse depolarization in normoxia (21% O_2) and 2 min after perfusion with hypoxia-equilibrated solution (10% O_2). Representative single channel recordings for each P_{O_2} conditions are shown in the top part of Fig. 3B. The corresponding ensemble-averaged currents are shown at the bottom. The effect of hypoxia was manifested by inhibition of the ensemble-averaged current amplitude, which was due to a decrease in open probability ($N \times P_O$) with no change in conductance. The effect of hypoxia on the K_{O_2} channel in PC12 cells as well as carotid body type I cells is manifested by a similar mechanism involving a decrease in open probability (P_O), a slowing of the activation kinetics, and little effect on channel closing (Ganfornina and López-Barneo, 1992; Conforti and Millhorn, 1997) Further molecular biological experiments have suggested that the K_{O_2} channel in PC12 cells belongs to the Kv1 subfamily of voltage-dependent K^+ channels (Conforti and Millhorn, 1997).

3. Ca^{2+} homeostasis

Inhibition of the K_{O_2} current in PC12 cells results in membrane depolarization and consequently an increase in intracellular Ca^{2+} (Zhu et al., 1996). The effect of hypoxia on membrane potential and Ca^{2+} mobilization in PC12 cells is shown in Fig. 4. Whole-cell current-clamp studies of membrane potential show that hypoxia causes membrane depolarization (Fig. 4A; normal resting membrane potential in PC12 cells is -35 to -45 mV) and that the degree of membrane depolarization is proportional to the severity of the hypoxic stimulus (Zhu et al., 1996). Hypoxia induces a 2–3-fold increase in intracellular Ca^{2+} in PC12 cells measured with Fura-2 dye (Fig. 4B). The increase in intracellular free Ca^{2+} returns to the control level upon re-exposure to normoxia. A similar effect of hypoxia on Ca^{2+} homeostasis has been reported for carotid body type I cells (Sterni et al., 1995; Bright et al., 1996). The hypoxia-induced increase in intracellular Ca^{2+} levels in PC12 cells is dependent on external Ca^{2+}, suggesting that the plasma membrane is the regulatory point for Ca^{2+} entry during hypoxia. In fact, exposure of PC12 cells to hypoxia in Ca^{2+}-free medium failed to induce

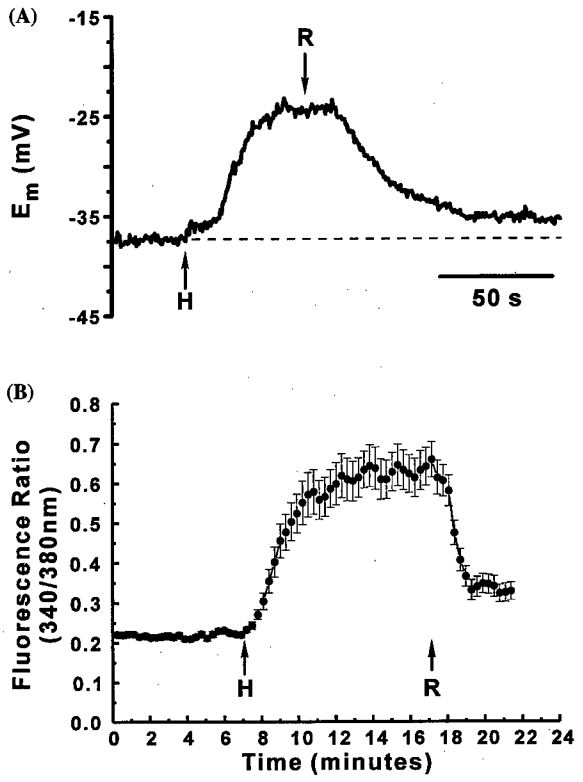


Fig. 4. Effect of hypoxia on resting membrane potential and cytosolic Ca^{2+} concentration in PC12 cells: (A) Membrane potential (E_m), recorded in whole-cell configuration in current-clamp mode; (B) Increase in cytosolic Ca^{2+} during hypoxia. Fura 2-loaded cells were imaged using 340:380 nm ratio method. Data are expressed as fluorescence ratio and represent average of 15 cells. Arrows indicate point of introduction of hypoxia (H; $P_{O_2} < 10$ mmHg) and return to normoxia (R; $P_{O_2} = 150$ mmHg).

any increase in intracellular Ca^{2+} (Raymond and Millhorn, 1997). The type of voltage-dependent Ca^{2+} channels responsible for the influx of Ca^{2+} during hypoxia has not yet been established. Incubation of PC12 cells with nifedipine or verapamil, known blockers of L-type Ca^{2+} channels, attenuates, but does not eliminate the increase in intracellular Ca^{2+} by hypoxia (Raymond and Millhorn, 1997), suggesting that the L-type Ca^{2+} channels contribute only partially to the increase in intracellular Ca^{2+} during hypoxia.

The increase in cytosolic Ca^{2+} by hypoxia mediates the release of DA, NE and other neurotransmitters from PC12 cells (Zhu et al., 1997; Kumar et al., 1998; Taylor and Peers, 1998). It has been shown that *N*-type Ca^{2+} channels play a critical role in neurotransmitter release during hypoxia in PC12 cells (Taylor and Peers, 1998). Although the release of neurotransmitters from PC12 cells during hypoxia and the role of Ca^{2+} influx through voltage-dependent Ca^{2+} channels in mediating transmitter release is well established, the specific type of voltage-dependent Ca^{2+} channels involved in the hypoxia-induced increase in intracellular Ca^{2+} and neurotransmitter release remains controversial (Kumar et al., 1998; Taylor and Peers, 1998).

4. Transmitter synthesis and gene regulation

The synthesis of DA is controlled by tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of catecholamines. Environmental hypoxia stimulates TH enzyme activity and gene expression in type I cells of the carotid body (Czyzyk-Krzeska et al., 1992). Similar responses to hypoxia have also been measured in PC12 cells (Czyzyk-Krzeska et al., 1994). Exposure of PC12 cells to 5% O_2 causes an increase in TH mRNA that is evident after 1 h exposure to hypoxia, reaches a peak at 6 h and persists at longer time of exposure (Fig. 5). The increase TH gene expression is due to both an increase in the rate of TH gene transcription and TH mRNA stability (Czyzyk-Krzeska et al., 1994). Nuclear runoff assays revealed that transcription of the TH gene is enhanced during 12 h exposure to hypoxia. The

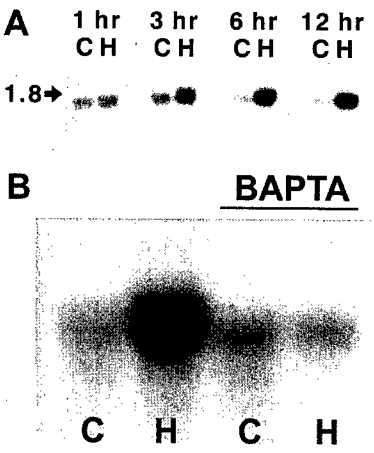


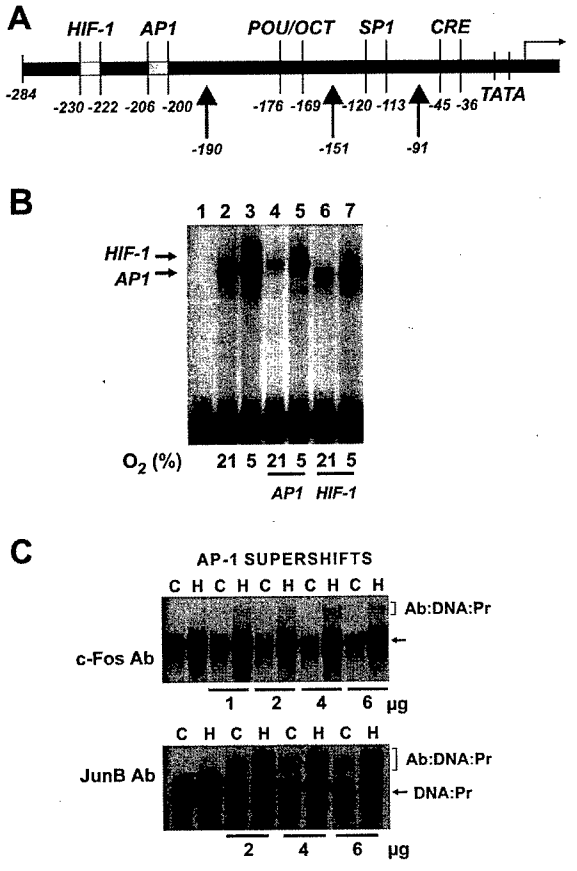
Fig. 5. Effect of hypoxia on steady-state levels of mRNA: (A) Northern blot of TH mRNA from PC12 cells maintained in normoxic incubator (C; 21% O_2) and cells exposed to hypoxia (H; 5% O_2) for 1, 3, 6 and 12 h; (B) Role of Ca^{2+} in the hypoxia-induced increase of TH mRNA. Tyrosine hydroxylase mRNA levels were measured in Northern blot experiments after incubation of the cells in either normoxia (C; 21% O_2), hypoxia (H; 6 h in 5% O_2) or hypoxia in presence of 100 μM BAPTA/AM.

maximum rate of transcription (4.5-fold above control levels) occurs 6 h after the onset of hypoxia, which corresponds to the maximum increase in TH mRNA levels (Czyzyk-Krzeska et al., 1994). Findings from experiments in which transcription was inhibited either with actinomycin D or 5,6-dichloro-1-D-ribofuranosylbenzimidazole, as well as pulse-chase experiments using 4-thiouridine showed that the half-life of TH mRNA was substantially increased during hypoxia (Czyzyk-Krzeska et al., 1994).

The signaling pathways that mediate TH gene regulation by hypoxia are still not fully understood. Recent evidence suggests that activation of TH gene expression during hypoxia is Ca^{2+}-dependent (Raymond and Millhorn, 1997). As discuss above, when PC12 cells are exposed to hypoxia, Ca^{2+} influx through voltage-dependent Ca^{2+} channels occurs (Fig. 4). The elevation in intracellular Ca^{2+} during hypoxia is important for inducing TH gene expression. Exposing PC12 cells for 6 h to hypoxia (5% O_2) results in a 3 to 4-fold increase in the steady-state mRNA level, as measured by Northern blot (Fig. 5B). This increase in TH mRNA is completely abolished by preincu-

bating the cells with BAPTA/AM, an intracellular Ca^{2+} chelator. Experiments using blockers of L-type Ca^{2+} channels, nifedipine and verapamil, only inhibited the hypoxia-induced increase in TH gene expression by 20% (Raymond and Millhorn, 1997). The specific Ca^{2+} channel subtypes that mediate the increase in intracellular Ca^{2+} responsible for TH gene regulation has not yet been characterized.

The mechanisms by which the increase in intracellular Ca^{2+} affects TH gene expression are still not well understood. It has been well established that elevation of intracellular Ca^{2+} induces expression of both the Fos and Jun family of transcription factors (Sheng et al., 1990; Passague et al., 1995). Previous studies have shown that the induction of the TH gene by hypoxia in PC12 cells is associated with nuclear protein binding to specific regulatory elements located in a region in the 5′ flanking region of the TH gene that extends from



– 284 to + 27 bases (Fig. 6; Norris and Millhorn, 1995). Results from chloramphenicol acetyltransferase reporter assays identified a region between bases – 284 and – 150 that contains *cis*-regulatory elements (AP1, AP2 and HIF-1) that are activated by environmental stimuli to regulate transcription (Fossom et al., 1992; Semenza and Wang, 1992). This region of the TH gene contains a number of other well characterized transcriptional regulatory elements, such as POU/OCT, SP1 and CRE. Gel mobility shift assays showed that AP1 and HIF-1 are the *cis* elements that interact with hypoxia-induced protein factors (Fig. 6B). In particular, supershift assays revealed that c-Fos and JunB binding to the AP-1 element is increased during hypoxia (Fig. 6C). The importance of c-Fos for functional activation of AP-1 and subsequently of the TH gene during hypoxia is further supported by experiments using c-*fos* antisense and mutations at AP-1 binding sites in the TH promoter region. Both c-*fos* antisense and mutation of the AP1 element in the TH gene results into loss of hypoxia-induced TH promoter activity in PC12 cells (Norris and Millhorn, 1995; Mishra et al., 1998). Expression of c-Fos and JunB in PC12 cells is

Fig. 6. Induction of protein binding to the 5′ promoter region on the TH gene by hypoxia: (A) Region of the 5′ promoter of the TH gene that extends from – 284 to + 27 bases and that contains various regulatory elements; (B) Gel shift assay using nuclear protein extracts from PC12 cells exposed to normoxia (C; 21% O_2) or hypoxia (H; 5% O_2). The TH DNA fragment corresponding to the sequences from – 284 and – 190 (containing the AP1, AP2 and HIF-1 elements) was used as a probe. Arrows indicate the protein-DNA binding complexes. Lane 1, in the absence of nuclear protein extract there is no shift in probe motility. Lanes 2 and 3, in the presence of nuclear protein extract hypoxia induces protein binding to this sequence of the TH promoter. Lanes 4 and 5, in the presence of nuclear protein extracts and excess nonlabeled oligonucleotide containing the AP1 sequence the binding activity is reduced. Lanes 6 and 7, in the presence of nuclear protein extracts and excess nonlabeled oligonucleotide containing the HIF-1 sequence the binding activity is reduced; (C) Presence of c-Fos and JunB in the AP1 binding complex revealed by supershift assays. Incubation of the gel shift binding reaction in normoxic and hypoxic cells with c-Fos (top) or JunB (bottom) antibody produces a supershift band (antibody–DNA–protein complex, Ab–DNA–Pr) that migrates above the normal shifted complex (arrow). The amount of Ab used is indicated at the bottom of each blot.

induced by hypoxia, as suggested by the supershift experiments where the c-Fos and JunB were detected in hypoxic PC12 cells (Fig. 6C. We did not identify the protein(s) that bind to the HIF-1 sequence.

Recent data show that hypoxia induces phosphorylation of the cyclic AMP response element binding protein (CREB) at Ser^{133}, an event that is required for CREB-mediated transcriptional activation (Beitner-Johnson and Millhorn, 1998). The CREB transcription factor regulates expression of many CRE-containing genes, including TH, c-*fos* and somatostatin (Sheng et al., 1990). The hypoxia-induced phosphorylation of CREB was more robust than that produced by either forskolin or depolarization. Furthermore, the phosphorylation of CREB by hypoxia occurs via a novel signaling pathway, in that it is not mediated by any other signaling pathway known to induce CREB phosphorylation (Beitner-Johnson and Millhorn, 1998).

5. Transmitter release

5.1. Feedback modulation of cell excitability by DA

The role of the released neurotransmitter in the process of chemosensitivity is still poorly understood. It was recently reported that the DA released by glomus cells of the carotid body regulates cell excitability by feedback inhibition of Ca^{2+} currents (Benot and López-Barneo, 1990). Our laboratory has studied the autoregulatory effect of DA released by hypoxia in PC12 cells (Zhu et al., 1997). High-performance liquid chromatography (HPLC) studies showed that 1–3 h exposure of PC12 cells to 5% O_2 results in a two-fold increase in DA secretion (Fig. 7A). The release of DA from PC12 cells upon hypoxia was later confirmed by others using amperometric as well as HPLC techniques (Kumar et al., 1998; Taylor and Peers, 1998). Interestingly, the release of DA from PC12 cells is also evoked by exposure to TEA (Taylor and Peers, 1998). This suggests that inhibition of the TEA-sensitive K_{O_2} channel in PC12 cells is a very early event in hypoxia-induced release of neurotransmitter in PC12 cells (Zhu et al., 1996). PC12 cells express D_2 receptors, as shown by RT–PCR experiments (Fig. 7B). Stimulation of the D_2 DA receptor by the DA agonist quinpirole resulted in a marked inhibition of the Ca^{2+} current (Fig. 7C). This effect is independent of protein kinase A (PKA), and appears to be mediated by $G_{i/o}$ proteins that couple the D_2 receptor to the Ca^{2+} channel (unpublished observations). We therefore hypothesized that the activation of the D_2 receptor would lead to a reduction in the increase in intracellular free Ca^{2+} during hypoxia (Fig. 7D). Indeed, hypoxia causes an increase in intracellular free Ca^{2+} that is significantly reduced by addition of quinpirole. The quinpirole-induced inhibition of the enhanced level of cytosolic free Ca^{2+} during hypoxia is abolished by the presence of the D_2 receptor antagonist sulpiride. Therefore, one role for DA that is released during hypoxia is 'feedback' regulation of cellular excitability by modulating the increase in intracellular free Ca^{2+} (Zhu et al., 1997). Thus, released DA might exert a negative feedback control over important cellular functions that depend on elevated intracellular Ca^{2+} such as neurotransmitter release and activation of second messenger pathways involved in O_2-mediated gene regulation (Raymond and Millhorn, 1997).

5.2. Feedback modulation of cell excitability by ADO

Adenosine is an endogenous metabolite of ATP that is produced and released in various tissues in response to a number of physiological and pathophysiological conditions including hypoxia (Winn et al., 1981). The cellular response to ADO is mediated by stimulation of different receptor subtypes (Fredholm, 1995). These are classified as A_1, A_{2A}, A_{2B} and A_3 (Dalziel and Westfall, 1994). PC12 cells express only the A_{2A} and A_{2B} receptor subtypes, but not the A_1 and A_3 receptors (Hide et al., 1992; Van der Ploeg et al., 1996; Kobayashi et al., 1998). Expression of A_2 receptor subtypes is shown in Fig. 8. In addition, RT–PCR data have shown that PC12 cells express adenosine deaminase, the enzyme that catalyses the first step of ADO degradation (Kobayashi et al., 1998). The presence of this enzyme suggests that PC12 cells produce ADO. HPLC experiments have shown that ADO

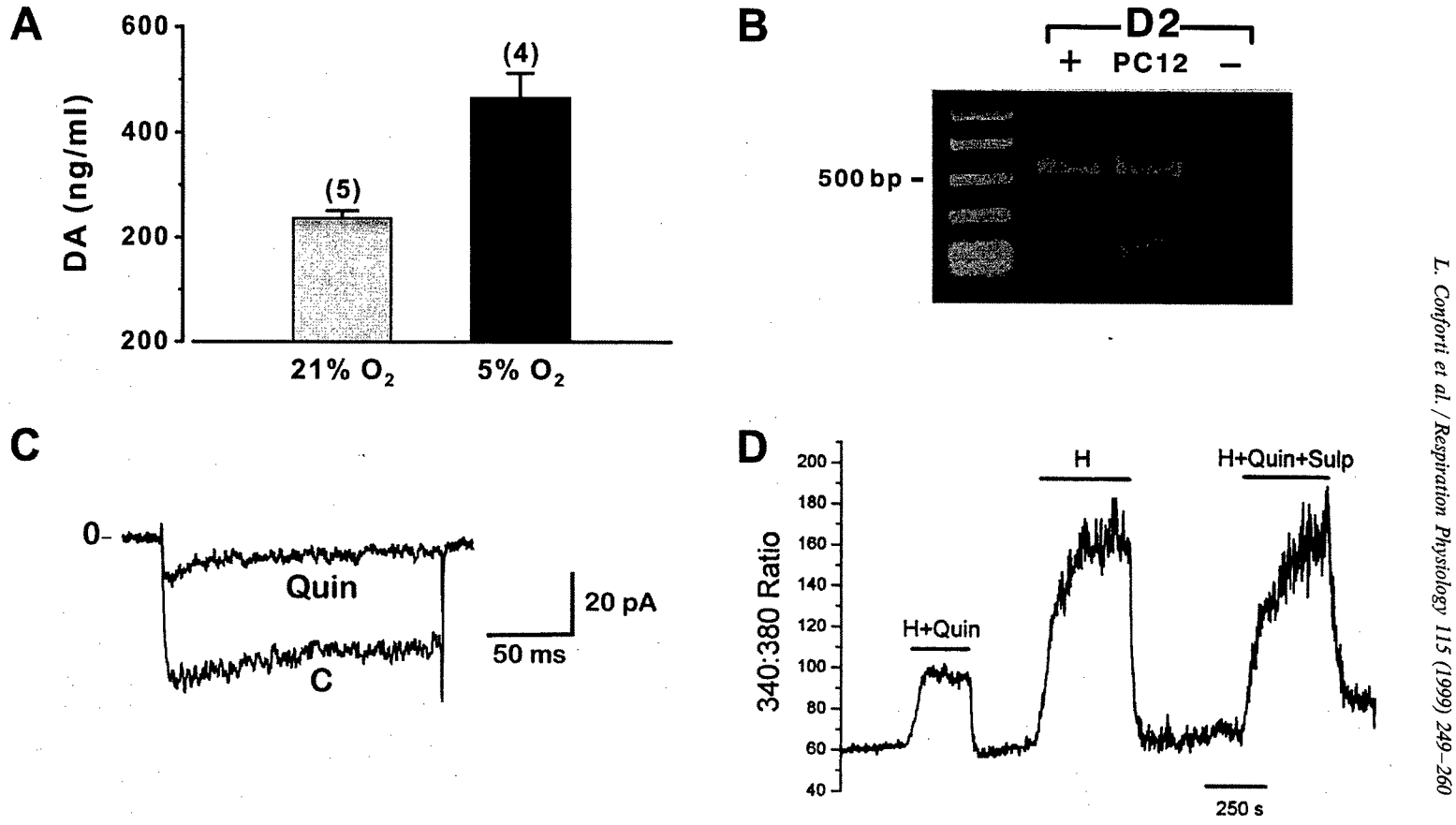


Fig. 7. Effect of DA on PC12 cell function: (A) Release of DA from PC12 cells during exposure to hypoxia. Levels of DA in the culture medium in cells incubated in normoxia (21% O_2) or hypoxia (5% O_2, 1–3 h) were measured by HPLC; (B) Expression of DA D_2 receptor mRNA in PC12 cells. Ethidium bromide visualization of PCR products obtained by RT–PCR of total RNA of PC12 cells and rat whole brain (positive control, +). PC12 cell total RNA that undergo PCR without RT is used as negative control (−) to assess for genomic contamination. The far left lane corresponds to the Promega PCR marker which contains 6 DNA fragments of 1000-50 bp. The D_2 receptor gene is expressed in both brain and PC12 cells (533 bp). The set of primers used was previously described (Zhu et al., 1997); (C) Effect of D_2 stimulation on Ca^{2+} current. Ca^{2+} currents are evoked by step depolarization from −80 mV HP to +20 mV (160 ms duration). Ba^{2+} (20 mM) is used as charge carrier and experiments are performed in Na^+- and K^+-free external and pipette solutions. Superimposed currents are recorded before (C) and after application of 10 μM quinpirole (Quin); (D) Effect of D_2 stimulation on Ca^{2+} mobilization. The effect of hypoxia (0 mmHg) on the fluorescence ratio at 340/380 nm is measured in PC12 cells loaded with Fura-2. Cells are exposed to hypoxia in presence of 10 μM quinpirole, hypoxia alone and hypoxia in presence of 10 μM quinpirole and 10 μM sulpiride consequently.

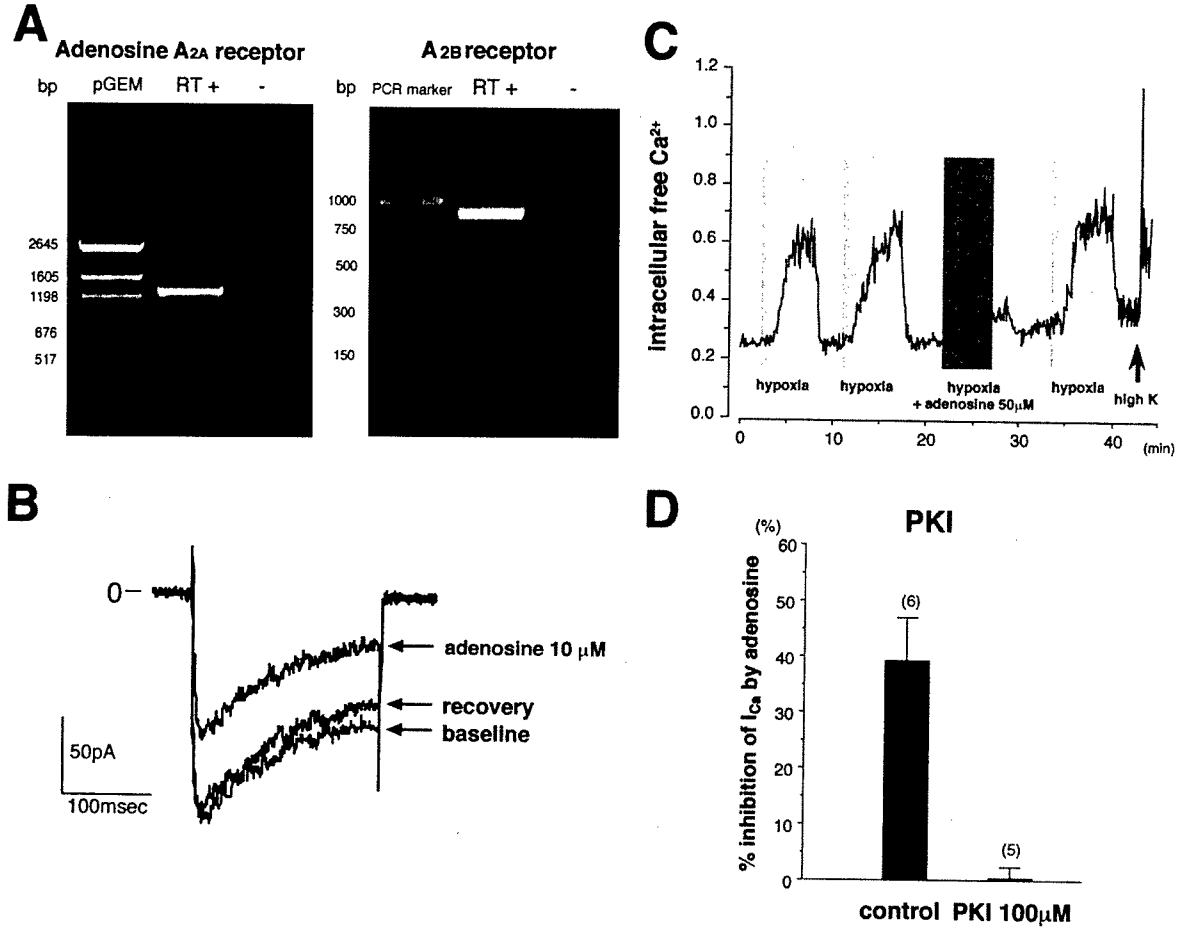


Fig. 8. Effect of ADO on PC12 cell function: (A) Expression of ADO A_{2A} and A_{2B} receptor mRNA in PC12 cells. Ethidium bromide visualization of PCR products obtained by RT–PCR of total RNA of PC12 cells (RT^+). Total RNA that underwent PCR without RT is used as negative control (−). The far left lane corresponds to the DNA marker. The A_{2A} and A_{2B} receptor genes are both expressed in PC12 cells (1320 bp for A_{2A} and 885 bp for A_{2B}); (B) Effect of ADO receptor stimulation on Ca^{2+} current. Ca^{2+} currents are evoked by step depolarization from −80 mV HP to +20 mV (160 ms duration). Ba^{2+} (20 mM) is used as charge carrier and experiments are performed in Na^+- and K^+-free external and pipette solutions. Superimposed currents are recorded before (baseline) and after application of 10 μM ADO and after returning to drug-free solution; (C) Effect of ADO receptor stimulation on Ca^{2+} mobilization. The effect of hypoxia (0 mmHg) on intracellular Ca^{2+} concentration (measured as the fluorescence ratio at 340/380 nm in PC12 cells loaded with Fura-2) is measured in the absence or presence of 50 μM ADO and upon exposure to 30 mM KCl; (D) Protein kinase A mediates the response of Ca^{2+} channels to ADO. The inhibition of Ca^{2+} current by ADO is measured in cells that are dialyzed through the patch pipette (1–2 mΩ) with a solution containing 100 μM PKI and compared with control cells (no PKI in pipette solution).

is released from PC12 cells during hypoxia, and the amount of ADO released depends on the severity of hypoxic stimulus (Kobayashi et al., unpublished). It is therefore possible that ADO released from PC12 cells during hypoxia can act in an autocrine-like fashion to regulate the cellular response to hypoxia via the A_2 receptors. Stimulation of the A_2 receptor by ADO released from PC12 cells has been previously reported (Erny et al., 1981).

The effect of ADO on the cellular response to hypoxia in PC12 cells is shown in Fig. 8C. The increase in cytosolic free Ca^{2+} induced by exposure to hypoxia is abolished by the presence of ADO. The mechanism by which ADO causes attenuation

of the hypoxia-induced elevation of cytosolic free Ca^{2+} is unknown, but it may be due to an effect of ADO on voltage-dependent Ca^{2+} channels. These channels are known to represent the main pathway for Ca^{2+} entry during hypoxia (López-Barneo, 1996; Taylor and Peers, 1998). Evidence for such a mechanism in PC12 cells comes from studies which revealed that stimulation of the A_2 receptor by ADO results in a reversible inhibition of the voltage-dependent Ca^{2+} current, as shown in Fig. 8B). This effect was blocked by an A_{2A} receptor antagonist ZM241385, suggesting that the A_{2A} subtype is the receptor that mediates the effect of ADO on Ca^{2+} homeostasis during hypoxia (Kobayashi et al., 1998). Similar effects of A_{2A} stimulation on Ca^{2+} current in PC12 cells have been shown by other investigators (Park et al., 1998). The inhibitory effect of A_{2A} stimulation on Ca^{2+} current is mediated by the cAMP-PKA pathway. The inhibition of Ca^{2+} current by ADO was blocked by the PKA inhibitor fragment (6–22) amide (PKI) (Fig. 8D) and does not occur in mutant PC12 cells that are deficient in PKA, but express the A_{2A} and A_{2B} ADO receptor (A123.7; Ginty et al., 1991; Kobayashi et al., 1998). The A_2 receptors are known to couple to adenylate cyclase via Gs protein (Dalziel and Westfall, 1994; Fredholm, 1995). Overall, the inhibitory effect of ADO on Ca^{2+} current and Ca^{2+} homeostasis might serve as a protective mechanism against the toxic effect of excessive intracellular Ca^{2+} concentration.

Ca^{2+} homeostasis during hypoxia appears to be under the negative control of feedback pathways that are stimulated by both DA and ADO released during hypoxia. Although the effect of both transmitters is inhibition of the hypoxia-induced increase in intracellular free Ca^{2+} via inhibition of voltage-dependent Ca^{2+} channels, different signal pathways appear to be involved.

6. Summary

Hypoxia is a very complex patho-physiological stimulus that affects various membrane and intracellular pathways, which, in turn mediate different aspect of gene expression. Although much progress has been done in the field of O_2-sensing, still important questions remain unanswered. A top priority is to identify the possible O_2-sensor/s that allow immediate detection of changes in the environmental P_{O_2}. Different possible O_2 sensors have been hypothesized, such as the K_{O_2} channel itself, a membrane-bound protein that directly or through a signaling pathway interacts with the K_{O_2} channel, intracellular mediators (e.g. kinases) as well as changes in the cytoplasmic oxidative state (Acker, 1994; Weir and Archer, 1995; Bunn and Poyton, 1996; López-Barneo, 1996).

The cascade of early events triggered by activation of the O_2-sensor are well-defined, including inhibition of the K_{O_2} channel, membrane depolarization, increase in intracellular Ca^{2+} and increase in transmitter synthesis. A future challenge will be to determine how the coordinated cellular response to hypoxia is regulated, in other words to identify the signaling pathways involved in linking the K_{O_2} channel inhibition to the nuclear events that regulate changes in gene expression during hypoxia.

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